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List of abbreviations

•O2	Superoxide
AIEC	Adherent-invasive *Echerichia Coli*
AOM	Azoxymethane
APC	Aloficocianine
APC/Cy7	Aloficocianine- cyanine 7
APCs	Antigen presenting cells
BB	Brush border
CA	Caffeic Acid
CAPE	Caffeic acid phenetil ester
CD	Crohn's Disease
CGD	Chronic Granulomatous Disease
CRC	Colorectal cancer
CTT	Coton-top tamarin
DSS	Dextran sodium sulphate
eNOS	Endothelial nitric oxide
FITC	Fluorescein isothiocyanate
H&E	Hematoxylin/Eosin
HLA	Human leukocyte antigens
IBD	Inflammatory Bowel Disease
IFN	Interferon
IL	Inter-leucine
iNOS	Inducible nitric oxide
KO	Knockout
LK	Leukotriene

LRR Leucine reach repeat

mAb Monoclonal antibodies

MAP Mycobacterium avium paratuberculosis

MDP Muramyl dipeptide

MDR Multi-drug resistence

NADPH Oxidase Nicotinamide adenine dinucleotide phosphate-oxidase

NK Natural Killer cells

NF Nuclear factor kappa B

NO Nitric oxide

NOD2/CARD15 Caspase recruitment domain family member 15

Nox Nicotinamide dinucleotide phosphate oxidase

O_2 Oxygen

PAF Platelete activating factor

PBMCs Peripheral blood mononuclear cells

PBS Phosohate buffer saline

PE R- Phycoerythrin

PE/Cy7 Phycoerythrin- cyanine 7

PerCpCy5.5 Peridinin chlorophyll protein-cyanine 5.5

PGs Prostaglandines

RIPK Receptor interacting protein kinase

ROIs Reactive oxygen intermediates

ROS Reactive oxygen species

SNP Single nuclear polimorphism

SRB Sulforhodamine B

TGF Tumor growth factor

TLR Toll-like receptor

TNF	Tumor necrosis factor
UC	Ulcerative Colitis
WT	Wild-type

Chapter 1

Abstract

Introduction Inflammatory Bowel Disease (IBD) as a chronic disorder of the gastrointestinal tract depends on mutations in the NADPH oxidase complex, responsible for the production of reactive oxygen species (ROS). IBD is refereed as Crohn's disease (CD) and ulcerative colitis (UC). Ncf1-mutation in mice leads to deficiency in ROS, rendering them susceptible to autoimmunity. As in humans, Ncf1-mutation leads to the lack of ROS in B10.Q mice. Studies focusing on how ROS-deficiency and colon-inflammation lead to tumorigenesis are still missing, as well as effective treatments. Hence the aim was to study how the lack of ROS influenced chronic DSS-induced colitis and the disease evolution to colon-adenocarcinoma in Ncf1-deficient mice and assess the effectiveness of caffeic acid-derivatives in human peripheral blood mononuclear cells (PBMCs) and human colon adenocarciona cell lines.

Methods Colitis was induced in Ncf1-mutant (Ncf1*) and wild-type (WT) B10.Q mice by oral administration of 3% (w/v) DSS. Two different colitis-induction protocols were used: Protocol 1- mice were submitted to 7 days DSS-induction, followed by 21 days of resting on normal water. Protocol 2- mice were submitted to 7 days of DSS-induction, followed by 14 days of resting on normal water and a second 7 days DSS-cycle. Animals were monitored every 2 days for alterations of colitis-related clinical scores: weight loss, diarrhea, colorectal bleeding and survival. Five Ncf1* and WT mice were sacrificed at the end of each time point: before the experiment had started and at days 22 and 30. Tissues were collected for histopathological analysis and assessment of colon lenght and spleen weight. The caffeic acid-based drugs were tested in human PBMCs and C2BBe1 and WiDr human colon-carcinoma cell lines with 6h, 12h, 24h and 72h incubation periods.

Results Colitis showed severer clinical and histological results in Ncf1* than in WT mice, with lower inflammatory reparation together with high grade dysplasia and invasive adenocarcinoma. Caffeic-acid derivatives markedly inhibited C2BBe1 and WIDr cell proliferation and high concentrations together with longer incubation periods enhanced proliferation of immune adaptive effector T cells.

Conclusion We propose a new animal model as a novel tool to study chronic colitis-induced carcinogenesis, where the absence of ROS keeps iNOs overexpression in Ncf1* colon during the chronic process, which promotes the high grade dysplasia and adenocarcinoma formation. Caffeic-acid derivatives are presented as novel possible drug

candidates against carcinogenesis development, due their dual capacity to promote both cancer cell death and activation of the adaptive immune system cells.

Key-words IBD, colitis, immune response, dysplasia, colorectal cancer, caffeic acid-derivatives.

Introdução A doença inflamatória do intestino (DII), referida como Doença de Crohn (DC) e Colite Ulcerativa (CU), leva modificações crónicas permanentes da estrutura gastrointestinal. Apesar das comuns características clinicas e patológicas, alguns fatores chave levam a que seja feita uma distinção das duas doenças. A DC pode envolver qualquer região do trato gastrointestinal, enquanto que CU afeta geralmente o reto, os perfis de resposta imune inata também contribuem a separação das doenças, sendo a DC caracterizada por um perfil Th1 e Th17, enquanto que a CU por um perfil Th2. A DII surge de uma resposta imune inflamatória inapropriada, em hospedeiros geneticamente susceptíveis e está ligada a mutações no complexo da NADPH oxidase, responsável pela produção de espécies reativas de oxigénio (ROS). Em murganho assim como nos humanos, a mutação no gene Ncf1 leva a uma deficiência na produção de ROS, tornando-os susceptíveis à autoimunidade. O desenvolvimento de cancro colo-retal é a complicação mais séria associada à cronicidade da DII. No entanto ainda estão em falta estudos que se foquem em como o défice de ROS e a inflamação do colon levam ao desenvolvimento tumoral, assim como tratamentos eficazes. Desta forma o objetivo deste trabalho foi estudar como a falta de ROS influencia a colite crónica induzida por DSS e a evolução da doença em murganhos mutados no gene Ncf1. Além disso, propusemo-nos a testar a eficácia de compostos derivados do ácido caféico em células do sangue periférico humano e linhas humanas de adenocarcinoma do colon.

Métodos A colite foi induzida em murganhos B10.Q mutados no gene Ncf1 (Ncf1*) e em WT, através de administração oral de DSS 3% (p/v). Foram aplicados dois protocolos diferentes: 1- os murganhos foram submetidos a 7 dias de indução com DSS, seguidos de 21 dias de água normal; 2- os murganhos foram submetidos a 7 dias de indução com DSS, seguidos de 14 dias de água normal e um segundo ciclo de 7 dias de DSS. Os animais foram monitorizados de 2 em 2 dias para alterações clinicas relacionadas com a colite: perda de peso, diarreia, hemorragia colorretal e sobrevivência. Cinco murganhos Ncf1* e cinco controlos foram sacrificados, os colons foram removidos e corados com HE para análise histológica e avaliação do comprimento do colon e peso do baço, antes do início da experiência e nos dias 22 e 30. Os compostos derivados de ácido caféico foram testados em células humanas do sangue periférico e linhas celulares humanas de adenocarcinoma (C2BBe1 e WIDr) com incubações de 6h, 12h, 24h e 72h.

Resultados A colite mostrou ter resultados tanto clínicos como histológicos mais severos nos murganhos Ncf1* do que nos WT, apresentando para além de uma fraca reparação inflamatória o desenvolvimento de displasia de alto grau e adenocarcinoma invasivo. Os

derivados de ácido caféico inibiram marcadamente, com concentrações preferencialmente elevadas e ao fim de 72h de incubação a proliferação de células C2BBe1 e WIDr. Simultaneamente não se mostraram tóxicos para as células do sangue periférico e uma vez mais mostraram que elevadas concentrações e longos períodos de incubação favorecem a proliferação de células T efetoras do sistema imune adaptativo.

Conclusão Propomos um novo modelo animal como ferramenta para estudar a carcinogénese induzida através da colite crónica, onde a ausência de ROS mantém a sobrexpressão de iNOS no colon de murganhos Ncf1* durante o processo crónico, levando ao desenvolvimento de displasia de alto grau e subsequentemente à formação de adenocarcinoma. Os derivados de ácido caféico são apresentados como uma nova possível droga no combate à carcinogénese, devido à sua dupla capacidade em promover a morte de células cancerígenas e a ativação de células do sistema imune adaptativo.

Palavras-chave DII, colite, resposta imune, displasia. cancro colorrectal, derivados de ácido caféico

Chapter 2

Introduction

2. Introduction

2.1 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic relapsing idiopathic immunological disorder of the gastrointestinal tract, leading to long-term and occasionally permanent pathological modification of the gastro-intestinal structure [1, 2]. IBD encompasses as two major forms of bowel disease, Crohn's disease (CD) and ulcerative colitis (UC). Extensive evidence suggests that IBD arises from an inappropriate inflammatory immune response to intestinal microbes in a genetically susceptible host. Even though UC and CD have several common clinical and pathological characteristics, key features distinguish both diseases, suggesting that the main pathological courses in the two forms of the disease are distinctive [2-9].

Crohn's disease can involve every part of the gastrointestinal tract, but normally, the terminal ileum, cecum, peri-anal area and colon are the most affected regions. CD is characterized by the presence of normal segments of the bowel between affected regions. Histologically is characterized by transmural inflammation that may involve any part of the gastrointestinal tract, dense infiltration of lymphocytes and macrophages, presence of granulomas in up to 60% of the patients and fissuring ulceration [3, 4, 6, 8-12].

In Ulcerative Colitis, the inflammatory development involves the rectum and expands continuously until the proximal region. Histologically the inflammation involves superficial mucosal layers with infiltration of lymphocytes, granulocytes and loss of the goblet cells, presence of ulcerations and crypt abscesses can also occur [3, 4, 8, 11, 12].

2.1.1 Epidemiology

There is a bimodal distribution of the disease in the population, most individuals (up to 25% of the patients) [13] are diagnosed between ages 15 and 40 years, or in older individuals between 50-70 years old [3, 5, 14-17].

The colon is the frequent macroscopic site of disease in very young people, childhood-onset of UC is usually extensive, whereas adults are equally probable to develop UC restricted to the distal colon. CD occurring earlier to puberty affects most males, whereas adult females are more commonly affected [13].

The occurrence and prevalence of CD and UC varies around the world, but all races and ethnic groups are affected and the disease incidence is growing internationally [3, 16, 18]. In the absence of major genetic fluctuations by migration, alteration in the percentages in IBD

incidence within a country and the importance of the genetic factors, emphasize the magnitude of environmental factors in the pathogenesis.

It is known that ‘‘Westernization” of society is one of the reasons for recent increases in the incidence of IBD. Areas with the highest incidence and prevalence of IBD are Northern Europe and North America [3, 5, 13, 15, 19-22]. Besides westernization, there are other theories/hypothesis trying to explain the increase and the onset of the disease. The “cold chain hypothesis’’ proposes that refrigeration has gradually been changing the bacterial content of our diet, resulting in the increased growth of disease triggering organisms [13, 23]. ‘‘Hygiene hypothesis” offers an alternative for the increase of IBD, asthma, rheumatoid arthritis, and type I diabetes in our society. The theory proposes that contact with pathogens and parasites during childhood, stimulates protective immunity that will prevent later aggressive immunologic processes, meaning that a cleaner environment/excessive sanitation could reduce the exposure to environmental antigens to the point of weakening the maturation of the mucosal immune system and induction of immune tolerance [2, 5, 13, 20, 24, 25]. In spite of all the existing hypotheses to date, the most popular proposes that the onset or relapsing of IBD pathogenesis is due to the development of an extremely aggressive acquired immune response against a subset of commensal bacteria in a genetically susceptible host [2, 19].

2.1.2 Environmental triggers

Previous studies have implicated environmental factors as one of the causes in genetically predisposed hosts to IBD. The environmental triggers act in IBD by altering/disrupting the mucosal barrier integrity, changing immune responses, or the luminal microenvironment [2, 3].

Antibiotics and diet (mostly diets with high contents of refined sugar and low on vegetable and fibre) can modify the luminal flora and dietary additives like aluminum and iron have an already well-defined role in stimulating bacterial virulence [26, 27].

Nonsteroidal anti-inflammatory drugs and acute infections can cause inflammation, which results in increased mucosal permeability, leading to increase uptake of commensal bacterial antigens, that stimulate the T-cell-mediated intestinal inflammation [2, 3, 28].

Stress can alter mucosal permeability, mucosal blood flow, epithelial electrolyte, water secretion and expression of cytokines and neuropeptides. All referred factors seem to increase the likelihood of relapse in patients with inactive disease [2].

Smoking, in CD, can cause changes in blood flow and mucus secretion weakening the mucosal barrier, exacerbating disease development and leading to fistulas and strictures

formation, increasing the need for medication and accelerating the need for surgery [22, 29, 30]. Although smoking may anticipate a crises in CD's patients, it appears to be protective against UC [31]. In fact, nicotine patches are sometimes used for the treatment of UC. Experimental studies suggest that the beneficial effects of nicotine in UC are due to increased mucus production, decreased production of pro-inflammatory cytokines and nitric-oxide, and improvement of the intestinal barrier function, whereas nicotine's negative effects in CD seem to be related to an increased influx of neutrophils into the intestinal mucosa [2, 3, 5, 15, 20].

2.1.3 Luminal antigens/gut flora

Studies in several different animal models have demonstrated that luminal flora is required for IBD to develop in a susceptible host. Genetically susceptible animals that are maintained in a germ-free environment since birth, only develop colitis when they acquire luminal flora, needed for the immune system activation [5, 6, 32]. Studies using only one species of bacteria, *Bacteroids vulgatus*, were able to induce colitis in IL-$10^{-/-}$ susceptible mice [33]. The normal mucosal microflora is required to initiate and/or maintain the inflammatory process, by providing one or more antigens or co-stimulatory factors that drive the immune response [3].

Antigens of the microflora, are present in the body since the birth, so they might be subjected to an intra-thymic processing that allows the immune system to distinguish self-antigens from non-self-antigens, so the hypothesis that the organism could be reacting to this antigens and mounting an autoimmune response, suggests that the chronic mucosal inflammation of IBD may be considered an autoimmune disease [2, 4].

Although the gut microflora plays an important role in IBD, not all bacteria are potentially harmful, a class of organisms that are recently known as probiotics ameliorate inflammation as they are thought to work through the induction of suppressor cytokines [34, 35].

2.1.4 The presence of abnormal microflora

Some studies suggest that IBD is associated with pathologic organisms that establish a type of low-grade infection in the mucosa inducing the inflammatory response, other propose that IBD patients have a defective epithelial barrier that enables the proliferation of nonpathologic organisms, again promoting the inflammatory response [11].

A previous study confirmed the presence of higher amounts of mucosa-associated bacteria in biopsies from IBD patients when compared with control subjects [36]. Darfeuille-Michaud et al. also presented results on the increased mucosa-associated bacteria in IBD

subjects: 20-40% of CD's biopsy patients were associated with invasive *E. coli* against 6% of specimens from controls [37]. Fecal samples from CD patients show a reduced diversity when compared with healthy individuals. IBD patients exhibit a marked reduction in bacteria belonging to the *Clostridium coccoides* subgroup [38], and in particular *Clostridium leptum* [39]. High concentrations of fungal communities were repported in a study by Ott Kuhbacher et al. were they analyzed fecal biopsies from CD patients [40]. Studies using colonic *mucosae* cultures, revealed that CD disease patients have an increase content of bacteria compared to healthy individuals [41]. Another study by Favier , Neut et al. showed that bacterial enzyme activities, in particular b-D-galactosidase, were also decreased in fecal extracts from CD patients [42].

Even though some authors discredit that IBD might be caused due a specific pathogen, some pathogenic and potential harmful enteric bacteria are seen more frequently in IBD patients than in healthy individuals:

- ***Mycobacterium avium paratuberculosis (MAP)***, the causative agent of Johne's disease in cattle, which shares similarities to CD, characterized by granulomatous inflammation of the intestines [19, 43-46]. MAP can be cultured from blood of about 50% CD's patients and 22% of UC patients, but not controls [47]. Additional evidence demonstrated that T cells isolated from CD patients were very reactive to MAP, whereas this reactivity was absent in healthy control individuals [48]. Moreover, it has been shown that down-regulation of the autophagy gene, *IRGM*, alterations which are also connected to CD, leads to prolonged survival of MAP in macrophages [49].

- *Helicobacter*, cotton-top Tamarin colitis (CTT) is essentially the *Helicobacter* equivalent of MAP's Johne's disease but parallels with UC, with similar clinical and pathological findings [50]. A potential role for Helicobacter organisms is to orchestrate the change from a healthy microbiota to dysbiosis [43]. However, oftentimes the attempt to demonstrate their presence has failed.

- ***Escherichia coli*** have been isolated in increased numbers from CD's patients and demonstrated to have greater adherent capacity to human cells, when compared with those isolated from controls, and had the ability to disrupt the intestinal barrier by producing α-aemolysin [37]. These *E. coli* named adherent-invasive *E. coli* (AIEC) are able to invade intestinal epithelial cells and strain LF82 can invade human macrophages, survive and replicate for long periods [51]. Such infected macrophages release high levels of TNF-α, which is a key cytokine in intestinal inflammation and has been known to be released in large quantity in CD [35].

Complementary studies using molecular techniques to identify specific bacterial groups have also been applied to study bacterial microflora in IBD, however these studies do not support the presence of a specific, pathogenic organism in IBD [11].

2.2 Genetic

2.2.1 Familial aggregation in IBD

Epidemiological and family studies have provided great evidence that genetic factors have an important role in determining susceptibility to IBD. First-degree relatives of patients with IBD have a 4-to-20-fold increased risk to develop the disease. People with CD have a first-degree relative with the disease in 2% of the cases, and with IBD in 5% of the cases. Regarding people with UC, they usually have a first-degree relative with the disease in 5% of the cases, and with inflammatory bowel disease in 8% of the cases [15, 20, 52]. Despite the evidence supporting a genetic predisposition, most patients with IBD have no close relatives with IBD [3].

2.2.2 Disease aggregation in twins

The most compelling evidence of genetic influence in IBD comes from twin studies [53]. Monozygotic twins have a significantly concordance rate of 58% for CD, whereas only 10% for UC. Comparing with dizygotic twins the concordance rate is not significantly different from that from all siblings. Therefore it appears that the genetic contribution to the development of IBD is more important in CD than in UC [3, 15, 54].

2.2.3 Susceptibility genes involved in IBD

Thus far, there have been two general approaches for gene identification in complex genetic disorders, like IBD, the use of genome-wide linkage studies and the testing of candidate genes. The tendency appears to be that the implicated genes in IBD regulate several biologic functions, including immunoregulation, mucosal barrier integrity and microbial clearance and/or homeostasis [2]. Nowadays several regions on chromosomes, 1, 3, 5, 6, 12, 14, 16 and 19 have been renamed as IBD1–9 and in a few cases the gene or genes underlying the different chromosome loci that are linked to IBD were identified. However, the first and most promising candidate gene is associated with CD [20].

IBD-1: ***CARD15***, also known as *NOD2*, (caspase recruitment domain family member 15), is expressed in macrophages, dendritic cells, epithelial cells and paneth cells. [2, 4, 8, 55, 56].

There are three mutations/polymorphisms associated with NOD2/CARD15, causing amino-acid substitutions, Arg702Trp and Gly908Arg and a frameshift 1007fs—found in *CARD15's* region, which encodes a leucine-rich repeat (LRR) responsible for bacterial recognition. At least one of these mutations is present in 25–35% of CD patients of European ancestry [57]. The LRR region binds to muramyl dipeptide (MDP), which is the biologically active half of the peptidoglycan, a ever-present cell-wall polymer found in almost all bacteria [58, 59]. The binding of MDP to CARD15 activates nuclear factor NF-κB through a receptor-interacting serine-theronine kinase-2 (RIPK2)- dependent signaling pathway, that forms part of a central signaling pathway that stimulates the transcription of multiple genes, that encode both pro-inflammatory and protective molecules [58, 60, 61]. The mutations Arg702Trp, Gly908Arg and 1007fs cause defective MDP binding [2, 9, 11, 55, 56, 62-64], and result in reduced macrophage activation by the NF-κB pathway and increased luminal bacterial populations [65]. This raised the question of how a mutation impairing NF-kB activation could lead to an increase in NF-kB-dependent inflammation. A study by Uehara, Yang et al. found two possible explanations. First, a host defense defect due to NOD2 impaired function allowed an increase in bacterial colonization of the gut wall leading afterwards to NF-kB stimulation by other mechanisms. Second, the ligand molecule for TLR2 can activate NF-kB independently of NOD2 [66]. NOD2 polymorphisms, when expressed within enterocytes and Paneth cells, are associated with lower production of anti-bacterial α-defensins [9]. This might lead to bacterial overgrowth and subsequent infection and chronic inflammation. Despite the presented facts, some studies suggest that NOD2 only plays a small role in the pathogenesis of the disease, because of its strongest association only with CD, also because the polymorphisms occur predominantly in patients with small bowel disease and are restricted to certain racial groups, which is indicative of the complexities of a multifactorial disorder [8, 55]. In patients with variant *CARD15*, homozygotes for this variant gene have a 20- to 40-fold increased risk of developing CD, whereas the heterozygotes have only 2- to 4-fold increased risk [8, 15]. Primary-monocyte-derived macrophages from patients with CD who are homozygous for the truncating mutation in the LRR sensor domain (Leu1007fsinsC) have a globally blunted transcriptional response to MDP. Otherwise, NOD2 frameshift-mutation knock-in mice have an enhanced response to MDP and are susceptible to dextran sodium sulphate (DSS) colitis [9, 67].

Regarding the linkage studies, no evidence was observed in CD-UC or UC- UC affected relative pairs, indicating that the susceptibility gene at IBD1 likely conferred susceptibility only for CD [67].

IBD-3, on chromosome 6p involving the major histocompatibility complex (HLA) has been implicated constantly for both CD and UC in various linkage studies. Data from linkage and epidemiologic studies estimate the contribution of the HLA region to overall genetic risk as 64% to 100% for UC and 10% to 33% for CD [68]. Furthermore, this region contains the TNF gene, for which functional promoter polymorphisms affecting TNF expression have been reported [69]. Three promoter polymorphisms (in the -1031C, -863A, and -857T regions) have been found to be associated with susceptibility and progression of CD in a Japanese population and between CD and the -1031C allele in a European population [15, 70].

ATG16L1 and IRGM, these are two genes involved in autophagy, a mechanism for clearing intracellular components, including organelles, apoptotic bodies, and microbes [71]. In mice with low expression of *ATG16L1*, the morphologic features and gene expression of Paneth cells are abnormal. *ATG16L1* carriers with CD also have abnormal Paneth cell morphology. In mice, ATG16L1 appears to regulate secretion of IL-1β and inhibit intestinal inflammation [8]. Short interfering (si)RNA studies have demonstrated that IRGM is required for mycobacterial immunity and may have an analogous role in the granulomatous response often observed in CD [9, 49].

NADPH oxidase, it is a complex formed by several accessory proteins, that produces ROS and plays an essential role in cellular response in microbial invasion. Genetic mutations in genes encoding components of the complex, result in both X-linked and autosomal recessive forms of chronic granulomatous disease (CGD), which often develops in intestinal inflammation that is histologically similar to Crohn's colitis. A single nuclear polymorphism (SNP) within the first intron of NCF4 (encoding $p40^{Phox}$ subunit of the complex) was identified as a CD-specific susceptibility gene [71]. In a recent study Muise et al. reported a novel missense variant in NCF2 (encoding $p67^{phox}$ subunit) in patients with very early onset IBD, which results in neutrophil dysfunction and susceptibility to CD. In their study Muise et al. also described novel associations of the NADPH oxidase complex gene *RAC2* with CD is associated with enhanced susceptibility to IBD [9, 56, 62, 72].

IBD-5: SLC22A4 and SLC22A5, are two functional variants of the organic cation transporters ONCTN1 and ONCTN2, that have been associated with CD also in association with CARD15 mutations [73]. Heterozygous carriage of the risk alleles increases the risk for developing CD 2-fold, whereas homozygous carriage increases risk 6-fold [15, 62, 74, 75]. However some investigators are still hesitant to identify the mutations in these genes as causative of CD [2, 56].

DLG5, which encodes a scaffolding protein, is important in maintaining the epithelial structure, so any genetic variants in DLG5 could interfere with the epithelial barrier [56]. Two haplotypes of the *DLG5 gene* were found by Stoll et al. and have been associated with CD and UC. The variant 113G>A in association with *CARD15* mutations in patients with CD has been confirmed [76].

MDR1, the multidrug resistance gene variants have been associated with UC and CD [77, 78]. The MDR1 gene encodes P-glycoprotein 170, an efflux pump of amphipathic toxins, and is highly expressed at the apical surface of epithelia of the colon and distal small bowel. MDR1 is of particular attention because it has been linked with treatment-refractory IBD, and because $MRD1^{-/-}$ mice do not develop colitis [79].

PPARG, polymorphisms were related with human CD [80]. PPARγ acts as a nudear receptor that inhibits NF-κB activity and in active UC patients its expression is decreased [81]. Polymorphisms in PPARγ improve recruitment and retention of effector macrophages, neutrophils and T cells into the inflamed intestine [2].

MUC2, it is a major goblet-cell-derived secretory mucin and it is proven to be differentially expressed in human IBD, based in a study by Van der Sluis et al., where he reported that $MUC2^{-/-}$ mice display impaired goblet cells and develop spontaneous colitis, proving that MUC2 cells have a critical role in colonic protection [82].

2.3 Immune Response

Both CD and UC patients have activated innate (macrophage and neutrophil), acquired (B and T cell) immune responses and loss of tolerance to enteric commensal bacteria, either because of dysfunction in the primary or secondary mechanisms that normally drive and regulate such responses, or because of some dysfunction in the intestinal epithelial barrier that leads to inappropriate penetration of microbial antigens [2, 6]. CD and UC are both

characterized by enhanced recruitment and retention of effector macrophages, neutrophils and T cells into the inflamed intestine, where they are activated and release pro-inflammatory cytokines. Accumulation of effector cells in the inflamed intestine is a result of enhanced recruitment as well as prolonged survival caused by decreased cellular apoptosis [2].

2.3.1 Innate immune responses

Macrophages and dendritic cells in the *lamina propria* are increased in absolute number and have an activated phenotype in both forms of IBD, but have been studied in greater detail in CD. Production of pro-inflammatory cytokines and chemokines is enhanced in IBD, and expression of adhesion molecules and co-stimulatory molecules is also increased. Cells involved in innate immune responses are activated and the expression of most pro-inflammatory cytokines and chemokines are upregulated in both CD and UC. Activation of NF-κB stimulates expression of numerous molecules relevant to the pathogenesis of IBD. These include molecules involved in the inflammatory response, such as IL-1β, TNF, IL-6, IL-8 and other chemokines, ICAM1 and other adhesion molecules, and co-stimulatory molecules, including CD40, CD80, CD86 and the inducible T-cell co-stimulator ICOS. Expression of each of these pro-inflammatory molecules is increased in active IBD. By contrast, cytokines that induce TH1 and TH17 responses are selectively up regulated in active CD but not in UC. Selective inhibition of most of this cytokines attenuates the onset of experimental colitis [2]. Neutrophils cause tissue damage, which seems to be exacerbated in IBD patients due to an extended lifes-pan from impaired apoptosis, through the release of nonspecific inflammatory mediators, such as reactive oxygen intermediates, lipid mediators and proteases, and secrete cytokines like IL-1β and TNF-α [43].

2.3.2 Adaptive immune T cell responses

In contrast with what happens in the innate immune response, T-cell responses vary greatly between CD and UC, so they should be considered separately [4].

2.3.2.1 Crohn's disease

A variety of T-cell defects have been observed in IBD patients, this include, defective T-cell apoptosis, which has been associated with a rapid cell cycle in CD; defects in regulatory T-cell activation and function; and mouse models have demonstrated that both excessive pro-inflammatory and deficient anti-inflammatory responses may manifest [43].

Most mouse models present a similar cytokine profile to the one seen in the human disease. The Th1 traditional cytokine profile is dominant in patients with CD, and is mediated

by IFN-γ, the production of which is stimulated by IL-12, produced by antigen-presenting cells (APCs). CD can also present a Th17 cytokine profile, where IL-17 mediates Th17 responses. The production of this cytokine is stimulated by the production of IL-6, TGF-β and IL-23 by innate immune cells and APCs [43]. Additionally cytokines IL-27 and IL-21 are also increased in CD patients [2, 8, 9, 83].

2.3.2.2 Ulcerative Colitis

In UC there's also a range of T-cell defects that can be observed, defective T-cell apoptosis in UC has been associated with a slower than normal cell cycle and defects in regulatory cytokines, TGF-β or IL-10 are associated with the development of UC [43].

This disease is considered to have a Th2 cytokine profile, but the concentrations of IL-4 and IL-5 which are characteristically elevated in this type of response, have been variable in UC tissues. UC has been associated with the production of various autoantibodies, such as neutrophil cytoplasmatic antibody (pANCA) and anti-tropomyosin, which might be indicative of a Th2-mediated immune response. Studies on the production of immunoglobulin sub-classes, such as IgG1 and IgG4 predominate in UC. So, this way UC is considered to have an atypical Th2-type response, mediated by NK T cells that secrete IL-13 [2-4].

2.3.3 Adaptive immune B-cell responses

B cells produce antibodies to both bacterial and nonbacterial antigens in mouse and human IBD. This supports the hypothesis that, there is a break down in mucosal tolerance. Several antibodies against microbial products are increasingly recognized in IBD, this include anti-Saccharomyces cerevisae (ASCA), anti-12, anti-Ompc and anti-flagellin antibody CBir1, being that 78% of patients react at least to one of these antibodies [8]. This implies that rather than a global loss of tolerance, different patients may form one or more specific antibodies, and the combinations present may help stratify disease subtypes [43].

2.3.4 Malfunction of the immune system

It is accepted that IBD results from an inappropriate response of a defective mucosal immune system. Experimental evidence from studies in vitro, in animals and in humans suggest that several pathways might result in inflammatory cascades triggered by the microbial antigens [20].

First, the epithelial barrier is leaky in IBD patients. Several studies have shown a lowered epithelial resistance and increased permeability of the inflamed and non-inflamed mucosa in CD [84]. Defective regulation of tight junctions might one of the causes of increased

permeability [8, 11], it may be inherent or induced by infection or nonsteroidal anti-inflammatory drugs [43]. The inflammatory response often results in continued epithelial injury, which causes erosions, ulcerations and a decrease in the production of defensins. It was shown that patients with CD exhibited an approximately 50% decrease in expression levels of α-defensin compared with controls [11]. The importance of the epithelial barrier in disease predisposition is supported by the finding of abnormal intestinal permeability in some first-degree relatives with CD [9].

Innate immune mechanisms of the epithelial layer are sometimes disturbed. Mucosal epithelial cells have a different pattern of TLR expression. TLR3 is significantly down regulated in active CD. By contrast, TLR4 is strongly upregulated [85]. An upregulation of NOD2 in epithelial cells, has also been reported, which might compromise the ability of the host to eliminate invasive and pathogenic microbes resulting in chronic inflammation [20]. Abnormalities of acute inflammation were found in patients with CD, these consisted of a failure of neutrophil accumulation and IL-8 production at sites of trauma in the bowel. These findings were not present in healthy controls. The problem with neutrophil accumulation in CD was shown to be due to the reduced secretion of IL-8 by macrophages [55].

Animal and *in-vitro* studies suggested that, there might be a loss of tolerance to enteric bacteria inducing Th1 and Th17, pro-inflammatory immune responses [11, 20, 43, 67].

IBD patients have disturbed clearance of auto-reactive T-cell populations. Due to a failure of central (thymic) and peripheral tolerance, activated T cells persist and do not undergo apoptosis [20]. Defective mucosal T-cell apoptosis is possible to play a key play role in IBD, support for this theory comes from studies that investigated the Bcl-2/Bax protein family, where expression levels of Bax were clearly reduced in inflamed UC colonic epithelium [67].

Balance between effector and regulatory T cells is disturbed in IBD. When the disease is active, effector T cells (Th1 and Th2) predominate over regulatory T cells [20]. The release of reactive oxygen and nitrogen metabolites, cytotoxic proteins, lytic enzymes, and cytokines by T cells leads to exaggerated death of enterocytes and pathological inflammation of the host tissue [67].

2.4 Reactive Oxygen Species

Reactive Oxygen Species (ROS) have been largely studied from different perspectives, some think they are harmful and cause innumerous diseases, others think they are essential and conserved during our evolution and others think ROS should be kept in a very tight control.

ROS are chemical oxygen species with one or more unpaired electrons that make them chemically reactive to other species. ROS include superoxide ($^{\bullet}O_2^-$), the hydroxyl radical ($^{\bullet}OH$), the hydroperoxyl radical ($^{\bullet}O_2H$), nitric oxide ($NO^{\bullet}$), and singlet oxygen (O_2) [86-89]. In the normal aerobic metabolism oxygen is the last receptor of electrons in mitochondrial respiratory chain, being in last term fully reduced to water. A small percentage of electrons do not travel to the end of the chain staying free to react with O_2 and form $^{\bullet}O_2^-$ [86, 87, 89, 90]. However mitochondria is not the only precursor for ROS, there are oxidase enzymes like NADPH in phagocytic cells and arachidonic acid metabolizing enzymes (e.g. cyclooxygenase and lipoxygenase) that also produce superoxide that serves as predecessor for other ROS [87, 91, 92].

ROS may play a far more integral role in the regulation of cellular and whole organism health, than was previously thought. It is becoming evident that under physiological conditions, ROS participate in crucial cellular events, functioning either directly as signaling molecules and/or indirectly by mediating global changes in the cellular redox status. Some of their down-stream targets include metalloenzymes and transcription factors that are sensitive to redox changes [89, 90, 93-95].

2.4.1 NADPH in ROS production

NADPH is a professional ROS producer, its role is only to produce ROS, and it is localized within the cell membrane being constituted by various subunits. In its constitution it has cytochrome *b*558, which consists of two subunits $gp91^{phox}$ (Nox2) and $p22^{phox}$. Upon cell activation, two cytosolic regulatory subunits $p47^{phox}$ and $p67^{phox}$, as well as two small G proteins Rac1 and 2 are translocated to the membrane and associated with the cytochrome *b*558 [86, 89, 91, 94].

This enzyme complex then generates superoxide by one-electron reduction of oxygen via its $gp91^{phox}$ subunit using reduced NADPH as the electron donor.

NADPH oxidase is a distinct enzymatic source of cellular ROS generation, because this enzyme is a “professional” ROS producer [96], whereas the other enzymes produce ROS only as by-products along with their specific catalytic pathways [94].

Several lines of studies have suggested that one important physiological function of NADPH oxidase in mammalian cells is the modulation of multiple redox sensitive intracellular signaling pathways by generating ROS molecules, including inhibition of protein tyrosine phosphatases, activation of certain redox-sensitive transcription factors, and modulation of the functions of some ion channels [94, 97].

Some authors think that certain stimuli like radiation, heavy metals and toxic chemicals lead to cellular stresses and suggest that NADPH oxidase may be an important component of the cellular stress signal transduction network. NADPH oxidase/ROS-mediated signaling might therefore represent a cellular “alarm system” that can alert the cells and prime them either to be adapted to the stress or to undergo apoptosis, being an ancient mechanism of multicellular organisms’ defense [91, 93].

Therefore, Nox-mediated redox signaling activation may have a critical role in coordinating the responses of the cell to deal with the adverse effects, either by activating stress kinases and promoting stress tolerance or by removing the seriously damaged cells by inducing apoptosis [89, 91].

2.4.2 ROS in Immunity

Nox-generated ROS can participate in immune function in a variety of ways, which are not mutually exclusive. First, the reactive oxygen itself or its by-products such as HOCl and peroxinitrite can directly oxidize biomolecules in invading microbes in a fairly non-specific manner, resulting ultimately in molecular damage and microbial cell death. Second, ROS can participate in signal transduction mechanisms linked to immunity and inflammation. This occurs through the selective oxidation of specific signaling enzymes/proteins that are linked to processes such as the secretion of cytokines or the activation of other killing mechanisms. Such signaling targets include transcription factors as NFk-B, signaling proteins such as protein kinases and phosphatases and ion and/or proton channels [93]. The first role to be definitively established for Nox-derived ROS was in innate immunity, mediated by professional phagocytes like neutrophils and macrophages [93]. In addition, macrophages produce large amounts of NO during phagocytosis; when NO reacts with superoxide, it generates the highly cytotoxic chemical species peroxinitrite (HONO) [93]. The activity of the phagocyte NADPH-oxidase also triggers opening of proton and potassium channels [93], that are proposed to change the ionic environment of the phagosome thereby activating microbicidal proteases and contributing to microbial killing [93]. Regardless of the precise mechanisms, it is clear from the inherited CGD that mutations resulting in defects in ROS generation by the respiratory burst oxidase are associated with an inability of phagocytes to kill bacteria and other microbes [93] convincingly demonstrating a role for the Nox2 system in innate immunity mediated by professional phagocytes [93, 98].

Neutrophils are the first line of defense with highly efficient bactericidal and tissue-toxic mechanisms in host protection including the phagocytosis of opsonized particles, degranulation with release proteases and the production of ROS. Among these functions,

production of reactive oxygen intermediates (ROIs), known as respiratory burst, is especially important in microorganism killing. Considering that efficient phagocytosis and the subsequent production ROIs play an important role in the intracellular killing of microorganisms by phagocytes, defects in one or both of these functions may lead to a deficiency in phagocytic function [98, 99].

So, undoubtedly ROS are essential for immune response and for its action destroying pathogens, there are opinions that its deregulation leads to oxidative stress and consequently to disease. However there are examples like CGD where dysfunctional ROS production leads to high inflammation levels and many complications.

2.4.3 ROS Imbalance

ROS has to be kept under very straight concentrations, if there is an imbalance between ROS production and scavenging we are towards oxidative stress. Situations like this might happen because of antioxidant decrease (e.g. mutations in antioxidant enzymes like superoxide dismutase, glutathione peroxidase) and ROS over-production (e.g. excessive activation of ROS producers) [86, 89]. This homeostasis is essential to healthy function, otherwise increasing scavenging mechanisms can occur, for example: tissue injury because of lipid peroxidation, DNA and protein damage or in last term cell death by apoptosis or necrosis [86].

ROS can start to interact with DNA, lipids, proteins and those interactions can lead to diseases like cancer, heart disease and many others [86]. For example lipids oxidation can occur in the presence of free oxygen radicals, leading to the formation of hidroperoxides and lipid peroxides. Lipid peroxides and oxygen radicals are responsible for many of the damaging reactions in the cell. They stimulate the peroxidation reactions that are toxic to cells and cell membranes. They can damage biological membranes, make the membrane leaky, and eventually cause complete membrane breakdown [86, 87]. Another redox-sensitive transcription factor is p53. It was demonstrated that oxidation of p53 alters its conformation and disrupts its DNA binding activity, resulting in a pattern change of p53-dependent gene expression. Moreover, p53 was shown to act as a homeostatic regulator by lowering ROS levels in stem cells and controlling hematopoietic stem cell self-renewal. Finally, the NF-kB signaling pathway is also significantly altered by deregulated ROS. ROS activates NF-kB signaling through elimination of the IkB inhibitor [89]. An increase in ROS levels induces the activation of the IkB kinase (IKK), which in turn phosphorylates IkB, leading to its proteasome-dependent degradation [92, 100].

2.4.4 ROS in Disease

Regarding the importance of redox in regulating crucial cellular events, the presence of oxidative stress means that there are oxidative perturbations, that if not corrected could result in a disease state. Cancer, cardiovascular disease, and metabolic disorders such as metabolic syndromes are the leading causes of death worldwide, with the number of cases for each condition projected to increase substantially over the next few decades. Cancer is an - example of a complete disarray of cellular redox homeostasis. Unlike metabolic disorders, both oxidative and reducing cellular environments appear to play a role in carcinogenesis. Oxidative imbalances may trigger a multitude of molecular and cellular events induced by the ROS [89, 90].

2.4.4.1 Inflammatory Bowel Disease

Oxidant stress is a major factor in IBD. It is been described that IBD patients have an enhanced production of reactive oxygen metabolites by epithelial and phagocytic cells (neutrophils, monocytes and macrophages) that leads to an increased oxidative stress in mucosal tissues [87, 101, 102]. It is stated that monocytes and polymorphonuclear cells in IBD are activated and produce high levels of pro-inflammatory mediators such as leukotriene B4 (LTB4) or platelet activating factor (PAF) leading to the release of large amounts of potentially cytotoxic reactive oxygen metabolites demonstrated through *in situ* quantification and through evaluation of lipid peroxidation [87, 101, 103, 104]. This was also related to the augmented migration of neutrophils and macrophages into the bowel mucosa, higher levels of ROS and the degree of inflammation and tissue damage [87, 101]. It is been showed that not only there is an increased oxidative stress in mucosa but also a decrease of antioxidants, with low blood levels of vitamin C and vitamin E, low levels of CuZn superoxide dismutase (SOD), glutathione peroxidase, catalase, vitamin A and β-carotene, thus leading to an increased oxidative state [87, 102]. Clinical data have shown that the administration of bovine CuZnSOD leads to an attenuated mucosal inflammation and injury in Crohn's patients [87, 105]. It is also acknowledged that the use of sulfasalazine (SAZ), mostly known by its commercial name 5-ASA, is beneficial in CD by inhibiting cyclooxygenase and lipoxygenase activities, and interacts with the superoxide, suppressing the formation and promoting of the degradation of free radicals, therefore protecting from oxidative stress-induced mucosal injury and inflammation [87, 106, 107].

Pavlik *et al* say that besides ROS overproduction, NADPH is not involved in it, once that inhibiting it made no alteration in the susceptibility of mice to DSS-induced colitis, indicating that NADPH oxidase is not involved in the pathophysiology of this model of UC [108]. Leoni G.

et al demonstrated that epithelial ROS induce a pro-resolution of epithelial wound closure, by mediating Anexin 1 (anti-inflammatory protein) production that control excessive immune cell trafficking by many mechanisms, one of them neutrophil influx inhibition and apoptosis at resolving inflammation site [109]. So, the effective role of ROS in IBD is still not clear, however new lines of thought consider that ROS has a resolution role in IBD.

Inflammation is associated with the production of ROS as a probable cause of neoplasic evolution [110]. ROS is being reported to be cause of DNA alterations through the augmentation of 8-hydroxyguanine (8-OHDG) production [102] in CD. In another study by D'Inca *et al* on UC, the high levels of 8-OHDG correlated with higher levels of ROS lead to an accumulation of oxidative DNA damage alongside with the progression of the disease and correlates with higher dysplastic lesions and possible implications for mutagenic and carcinogenic progression [111]. It is already being demonstrated that IBD patients can have a defective neutrophil respiratory burst [112, 113]. Despite early reports from genome-wide association studies (GWAS) indicating the NCF4 gene as susceptibility gene for CD [71, 114] a recent GWAS study did not corroborate these results [115]. Even so, in a study by Muise *et al* they not only replicate the association of NCF4 with CD previously described but also illustrated a novel associations of the NADPH oxidase complex gene RAC2 with CD and introducing a novel missense variant in NCF2 with very early onset IBD that leads to an neutrophil dysfunction and susceptibility to CD, thus indicating that the NADPH oxidase complex genes play a role in the pathogenesis of CD [116].

2.5 IBD-associated colorectal cancer animal models

Cancer is a growing health problem around the world and is one of the major causes of death after heart disease [117]. Nowadays there are over 10 million cases of cancer each year, being colon-cancer one on the top of the list [118, 119]. In the past decades studies on CRC have shown that its incidence has been increasing, becoming one of the most common and lethal cancers in Western countries [120] and this problematic is also a growing concern to Asian nations [121].

Colorectal cancer (CRC) development is the most serious complication associated with longstanding IBD. Increasing risk causes are the duration of the disease and the extent of colorectal involvement. Cancer development follows a sequence of no dysplasia, indefinite dysplasia, low-grade dysplasia, high-grade dysplasia and carcinoma. In IBD patients neoplastic lesions arise within areas of the *mucosae* that have been involved with colonic inflammation [122].

Currently there are many animal models for study IBD, but only a small number of this are appropriate to study the dysplasia cancer-sequence. The most widely used mouse model is with DSS. In most studies, normally to successfully induce a carcinogenic path is usually needed a long period with multiple cycles of DSS-induction, or the use of carcinogenic coadjuvants to diminish the exposure periods. Cooper HS. et al. described a four-cycle DSS induction to be associated with the development colitis-associated dysplasia and adenocarcinoma, with dysplasia incidence of 13.9% at 12 week of treatment [123]. Other studies combine DSS induction with a colon carcinogen promoter to achieve a higher incidence of colonic tumors in lesser time. Tanaka T. et al. and others demonstrated through the use of azoxymethane (AOM) plus DSS that, the amount of time needed to promote colon carcinogenesis in mice could be reduced, despite the treatments resulted in high incidence of colonic neoplasms it still took 20 weeks [124]. One hundred percent of colonic tumorigenesis is achieved when compared to 15%-20% when DSS is administered alone. Although results also showed that mice receiving only AOM did not developed dysplasias, suggesting that the dose of carcinogen used is insufficient to induce dysplasic lesions in absence of inflammation caused by DSS [125]. In a similar way the use of different heterocyclic amines have been proved to be important food-derived carcinogens with ability to promote colon-neoplasia when associated to DSS- induced colitis [124, 126]. Clapper ML et al. in their review mentioned that the existence of determined mutations like $APC^{+/Min}$ in association with DSS treatment accelerate the formation of colitis-associated dysplasia's and their progression to invasive cancers [127]. Other important colitis-associated dysplasia mutation is the p53 gene, described in the work of Hussain SP. et al. Loss of p53 function is an early and critical event in colitis-associated colorectal cancer, and DSS-treated $p53^{-/-}$ mice have significantly more lesions (cancers and dysplasias) when compared to their non-mutated counterparts [128-130]. In a study focusing in mouse-models related to the inflammation and colitis-associated dysplasia, Sturlan S. et al. showed that IL-$10^{-/-}$ mice spontaneously develop colitis which eventually leads to high grade dysplasias culminating in adenocarcinoma in tender age [131, 132]. They also observed high similarities in the histopathological pattern of colorectal cancers between IL-$10^{-/-}$ and IBD-associated carcinomas in humans [131]. Chemically-induced tumor incidence, transplanted tumor growth and lung foci formation are increased in IL-$10^{-/-}$ mice [133]. More specifically Betra et al. showed how AOM and DSS treatment promotes inflammation mediated colonic tumor growth in IL-10 KO mice [134]. Patients with mutated IL-10 signaling systems show early and aggressive development of systemic inflammation including IBD [132]. The development of adenocarcinoma in IL-10 KO mice seems to be associated with colonic bacterial infection, this causes injurious leakage of bacterial antigens into the mucosa, which causes an immune

response that in turn leads to the development of IBD and subsequently adenocarcinoma in the host [135]. Seamons A. et al. utilized DSS to induce inflammation and cancer without the use of an additional carcinogen, in TGF-β signaling-deficient $Smad3^{-/-}$ mice [136, 137], which are defective in one of the most common signaling pathways mutated in human colorectal cancer [138]. Previously they have demonstrated that development of colitis and colon cancer in $Smad3^{-/-}$ mice requires a trigger, such as infection with enterohepatic Helicobacter, to induce disease, and others have shown that DSS serves as a chemical trigger to induce colon cancer in $Smad3^{-/-}$ mice [139]. Regarding iNOS in carcinogenesis there have been several debates about its play in the pathology. It is well known that in patients with UC iNOS levels are overexpressed relating with a path to carcinogenesis [140-145]. Although Seril D. et al. introduces to this problematic in his work, using iNOS knockout mice, in a DSS-colitis associated carcinogenesis model, he demonstrated that there was no significant differences when compared $iNOS^{-/-}$ mice with the WT, this way reducing the importance of iNOS in the pathology and at the same time left indications that eNOS (endothelial NOS) could have a significant place in the pathology. In the same way other researchers focusing in iNOS deficiency/inhibition on experimental carcinogenesis also demonstrated a multiplicity of results. Rao CV. et al. attenuated an AOM-induced colon-carcinogenesis using a iNOS specific inhibitor [146]. Also using an $APC^{min/+}$ mice model the use of aminoguadinina NOS inhibitor and L-arginine deficient, exhibited decreased colon-carcinogenesis [147], being also reported by Kisley et al. 2002 that iNOS knockout mice model are resistant to carcinogenesis tumor lung-cancer [148]. However, another study by Scott DJ. et al. reported a slight enhanced in colon-carcinogenesis development in $APC^{min/+}iNOS^{-/-}$ [149].

2.6 Phenolic-acid derivatives

Nowadays innumerous cancer-treatments are available, nevertheless they still remain insufficient to treat/eradicate most cancers and/or inhibit cancer metastasis. Therefore, research for novel anticancer agents that are highly effective, with low toxicity improving the disease condition is constantly increasing [150]. A novel generation of natural products offers opportunities for innovation and discovery of new therapeutics. Natural products play a major role in cancer prevention and treatment, and currently a considerable number of this natural antitumor agents is already being used [151]. The protective effects against cancer found in the natural dietary compounds are believed to be due to the induction of cellular defense systems (detoxifying and antioxidant enzymes system), inhibition of anti-inflammatory and cell growth signaling pathways, culminating in cell cycle arrest and/or cell-death [152].

Natural compounds as plant polyphenols are always present in a diet rich in vegetables and fruits and are considered to be serious candidates for being responsible for cancer protective effects [153]. Dietary polyphenols are mainly consumed through fruits and beverages (juice, wine, tea, coffee and beer), apart from vegetables, cereals and olive derivatives, food components mainly associated to the mediterranean diet [154]. Polyphenolic compounds are a heterogeneous group of compounds that have been categorized based on their structure in phenolic acids and analogs, stilbenes, flavonoids and analogs, miscellaneous.

Propolis, is a natural resinous substance collected from trees by *Apis mellifera* honey bees by collecting resins and changed by the action of enzymes contained in their saliva [155-157]. Propolis has long being used in traditional medicine [158] and has been reported to have a broad spectrum of biological activities such as, anti-inflammatory, antioxidant and antitumor [158-161], due to its list of constituents, where we find 2-20% of caffeic acid (CA) and its esters [162], which are important phenolic acids with innumerous health benefits. Caffeic acid ester derivatives present in propolis are more lipophilic than CA and thus easily facilitate their entry into the cell, and also require small amounts to exhibit their effective inhibitory activities [163].

In 1993 Frenkel, Wei et al. worked on the antitumor/anti-inflammatory activity of propolis, showing that caffeic acid phenethyl ester-CAPE (synthesized by esterification of CA [164]) was a potent inhibitor of a number of oxidative processes at very low doses both *in vitro* and *in vivo* [165]. Jaganathan, Mandal et al. study in honey revealed that the sample exhibiting the highest phenolic concentration showed the best protective effects against oxidant-induced cell death [166]. Since the potential utility of caffeic acid ester derivatives in tumor inhibition, Rao Desai and its team studied the antimutagenic and antitumorigenic activities of several synthesized caffeic acid esters, were they significantly inhibited the growth of human colon HT-29 and HCT-116 cells and the activities of ODC (ornithine decarboxylase) and TPK (tyrosine protein kinases) at much lower concentrations when compared to CA. Results demonstrated that caffeic acid esters possessed antimutagenic activity against DMAB-induced mutagenecity *in Salmonella typhimurium strains TA98 and TA 100* [167]. In a follow up study Rao Desai et al. presented results on how the same caffeic acid esters derivatives present in honey possessed inhibitory activities against AOM-induced colonic ODC, TPK, and lipoxygenase activities and ACF formation, which are relevant to colon carcinogenesis [168]. The mechanisms of CA-induced apoptosis in colon cancer cells, was later studied by Jaganathan, Mandal et al. where CA notably inhibited HCT15 colon cancer cell proliferation in a dose-dependent manner. CA treatment increased the number of cells in sub-G1 phase of cell cycle, which indicates apoptosis [169]. CA inhibited colony formation similar to some anticancer agent like Triphala (TPL) [121]. In 2012 Dev-Aur Chou and its team reported the anticancer effects of caffeate

derivatives in COLO 205 human colon cancer cell line. Decyl caffeate induced apoptosis in COLO 205 cells through both Fas- and mitochondria-mediated pathways [170]. Research by Banskota, Nagaoka et al., using Netherlands propolis MeOH extract showed antiproliferative activity against highly liver-metaststic murine colon 26-L5 carcinoma cells [158]. Those results led the team to continue the research and further investigate the structure-activity relashionship of CAPE and twenty synthesized CAPE-analogues and examine their antiproliferative activities in six tumor cell lines, showing that of all the tested cell lines CAPE analogues possessed selective antiproliferative activity toward liver-metaststic murine colon 26-L5 carcinoma cell line [164]. The same CAPE-analogues also inhibited tumor nodules and metastasis in lung cancer [171]. Lee, Liao et al. reported the effect of nine synthesized CAPE-like analogues for investigating the growth of human neck cancer, revealing that CAPE and its ethyl analogues were considerable cytotoxic [172]. Previous studies on how CA significantly offers protection against UVB-induced erythema in humans and UVC-induced in cell lines [173, 174], justified a scientific investigation into the possible potential use of CA as a protective agent against UVB radiation-induced skin damages. Staniforth, Chiul et al. findings suggested that CA may prevent UVB-induced immune suppression by inhibiting IL-10 expression. While CA inhibition of UVB-induced MAPK signal transduction pathways and downstream transcription factors AP-1 and NF-kB may confer protection against photocarcinogenesis [175]. Kudugunti, Vad. and its team studied the effect of CAPE as anti-melanoma agent in skin tumor model of melanoma in C57BL/6 mice and found it to cause significant tumor growth inhibition when in B16-F0 melanoma cell line with minimal toxicity [176]. Research done on the role of CAPE and how it interacts with tumor necrosis factor (TNF), revealed that CAPE completely blocks activation of NF-kB by TNF in a dose-and time-dependent manner and the activation of NF-kB by other inflammatory agents is also blocked [177]. Few years later Mirella Nardini reported the effect of CA on Cer (ceramide)-induced NF-kB activation and apoptosis in the human monocytic U937 cells, the results indicated that very low concentrations of CA modulate Cer-triggered signal transduction pathway, including NF-kB activation and apoptotic response in U937 cells [178]. Further studies in which the effects of CAPE on iNOS expression and iNOS enzyme itself activity were examined, elucidated that CAPE is an inhibitor of LPS and IFN-Ɣ-induced NO production whose action is mediated by inhibiting iNOS gene transcription via action on the NF-kB site is the iNOS promoter as well as by directly inhibiting the catalytic activity of iNOS [179]. With regard to the synthesis of PGs, Masferrer et al. showed that CAPE inhibits TPA- and carrageenan-mediated induction of COX-2, directly inhibits the activities of COX-1 and COX-2, and inhibits the release of arachidonic acid from membrane phospholipids [180]. The effects of caffeic acid ester derivatives were studied on NO production in murine

macrophages RAW264.7 cells by koji Uwai et al. Several compounds with various lengths and forms of the alcoholic alkyl side chains were arranged and the results revealed that their inhibitory effect on NO was dependent on the lenght and size of the side chain [181]. Investigation on the cytotoxicity of novel lipophilic caffeic and freulic acid derivatives in human breast cancer cells also concluded that an alkyl chain through an ester or an amide bond on both caffeic and freulic acid increases the cytotoxicity toward the cancer cell line [182]. Further research on cancer angiogenesis relationship with CA and its derivatives revealed an inhibition in tumor growth and angiogenesis process by inhibition of STAT3 activity, expression of HIF-1α and VEGF in a mouse xenograft model [183].

2.7 Objectives

The present work aimed to study how the lack of ROS influenced chronic DSS-induced colitis and disease evolution to colon-adenocarcinoma in Ncf1-deficient and WT B10.Q mice. Afterwards, the quality of the results on the animal model have provided the basis for a follow-up project, where it was intended to unveil the synergies between the immune system and caffeic acid-based drugs in a combined therapy for colon-adenocarcinoma.

To achieve the proposed goals we:

- Evaluated the clinical evolution of experimental colitis;
- Evaluated the histopathological evolution of colitis;
- Cultured peripheral blood mononuclear cells with caffeic acid-derivatives;
- Cultured human colon-adenocarcinoma cell lines with caffeic acid-derivatives.

Chapter 3

Materials and Methods

3. Materials and Methods

3.1 Animals

Female and male 6-8 weeks old homozygous Ncf1-mutant (BQ.*Ncf1*$^{m1J/m1J}$, abbreviated Ncf1*, n=30) and wild-type (WT, n=30) B10.Q mice (C57BL/10 expressing H-2^{q}) were obtained from breeding heterozygous mice followed by genotyping, as previously described [184]. Animals were bred and maintained under standard conditions, with food and water *ad libitum* in a specific pathogen–free environment. All animal studies were approved by the internal CNC Ethics Committee, and were in accordance with EU legislation for experimental animal welfare.

3.2 Induction of colitis

Colitis was induced by oral administration of 3% (w/v) DSS (average 40,000 g/mol, AppliChem, Darmstadt, Germany) in the first induction cycle and 2.5% (w/v) DSS in the second cycle *ad libitum* in drinking water. Reduction of DSS concentration was to avoid premature death. Two different colitis-induction protocols were used (Fig.1): Protocol 1 (P1)- mice were submitted to 7 days DSS- induction, followed by 21 days of resting on normal water. Protocol 2 (P2)- mice were submitted to 7 days of DSS-induction, followed by 14 days of resting on normal water and a second 7 days DSS-cycle. Five Ncf1* and WT mice were sacrificed at the end of each time point: before the experiment had started and at days 22 and 30. Tissues were collected for histopathological analysis and assessment of colon lenght and spleen weight.

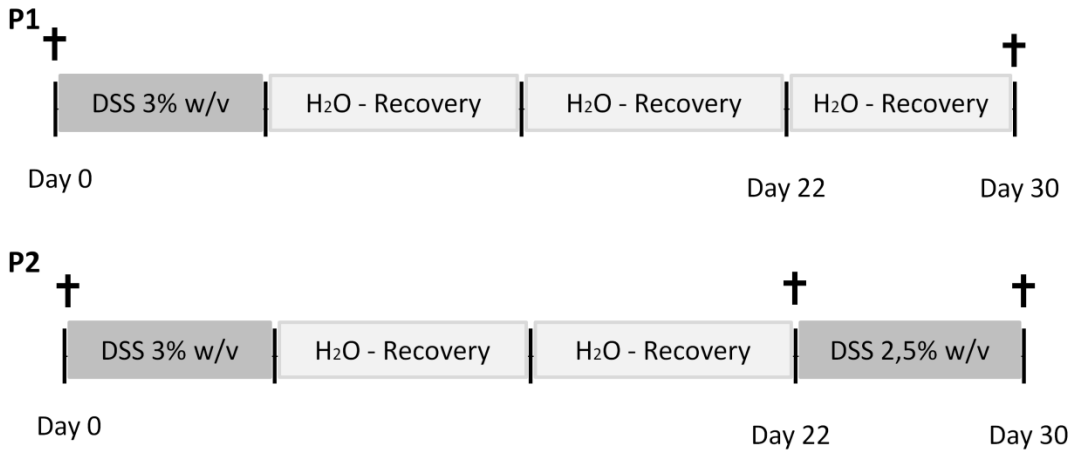

Fig.1 Schematic overview of DSS treatment plan. P1) Protocol 1: one week of DSS- induction, followed by 3 weeks of recovery. P2) Protocol 2: one week of DSS-induction, followed by 2 weeks of recovery and a second week of DSS-induction. Crosses (ǂ) indicate time-points where the mice were sacrificed.

3.3 Clinical evaluation of colitis

Animals were monitored every 2 days for alterations of colitis-related clinical scores: weight loss, diarrhea, colorectal bleeding and survival. Blood and stools scoring was performed as previously described [185]. Blood scoring: 0- no blood; 1- visible blood; 2- rectal bleeding. Stool consistency scoring: 0- Normal; 1- Soft but formed; 3- very soft; 4- diarrhea.

3.4 Histopathological evaluation of colitis

Swiss rolls for the whole colon, rolled from the rectum to the cecum, were formalin fixed, paraffin embedded and sections were hematoxylin/eosin (HE) stained according to standard protocols.

Inflammation was scored for proximal and distal segments regarding the number of inflammatory foci: 0- no inflammatory focus; 1- one inflammatory focus; 2- two inflammatory foci; 3- three or more inflammatory foci. Lymphocytes, plasma cells and neutrophils infiltration was scored based on cell morphology, with approximate percentage validation of each subset within the total observed inflammatory cells in inflammatory foci. Epithelial morphology was scored based on the presence of tubular and villous hyperplasia, either as glandular hyperplasia above the muscularis mucosae or as formation of superficial villous projections, respectively. Dysplasia was scored in the same segments using a semi-quantitative scale according to the WHO 2010 guideline for colon adenocarcinoma classification [186]: 0- no dysplasia; 1- hypercromatic nuclear pluristratification and lamina propria separated glands; 2- epithelial low grade dysplasia (complex ramified glands with cell hyperplasia and pluristratified hypercromatic nuclei); 3- epithelial high grade dysplasia (beyond low grade dysplasia, nuclear atypia and mitosis).

3.5 Caffeic acid-derivatives

Four caffeic-acid derivatives were used, ferulic acid (FA), feruloylhexylamide (FAHA) , caffeoylhexylamide (CAHA) and caffeic acid (CA) (Fig.2), they were synthesized as previously described [187], and ressuspended in Dimethyl sulfoxide (DMSO, Sigma Aldrich) to achieve 4mM. The compounds were kindly donated by the Drug Discovery group from Faculty of Pharmacy University of Coimbra.

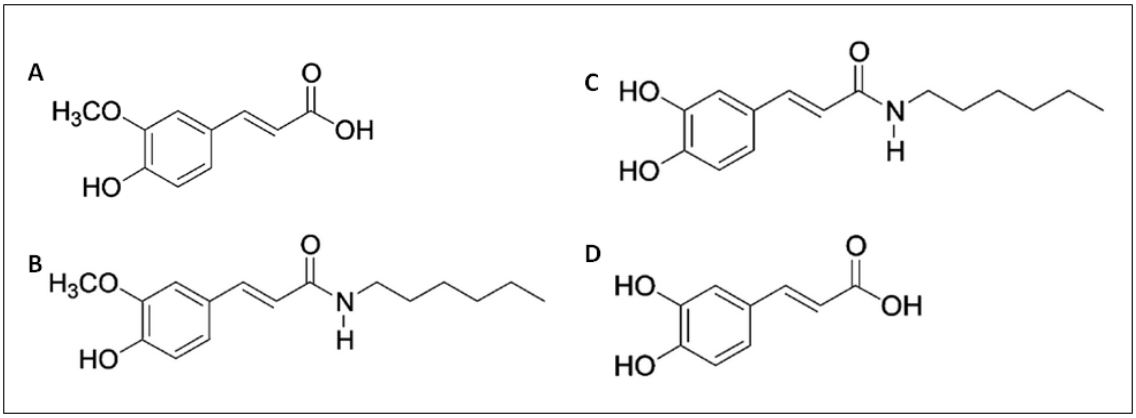

Fig.2 Chemical structure of the caffeic-acid derivatives. A) Ferulic acid (FA); B) feruloylhexylamide (FAHA); C) caffeoylhexylamide (CAHA) and D) caffeic acid (CA).

3.6 Cell culture

3.6.1 Peripheral Blood Mononudear Cells (PBMCs)

PBMCs were lysed with 1x RBC lysis buffer (NH4Cl 0.15M, KHCO3 10mM, EDTA 0.1mM), for 10min and then centrifuged at 1250rpm for another 10min, the process was repeated twice. Supernatant was discarded in between steps. Cell suspension was then washed with 1x phosphate buffer saline (PBS), ressuspended and cultured in RPMI medium (L-glutamine 2mM, HEPES-NA 10mM, penicillin 100U/mL, streptomycin 100ng/mL), supplemented with 10% heat-inactivated foetal bovine serum (FBS) at 37^{o} C in a humidified atmosphere containing 5% CO_2. An average of 250,000 cells/mL were plated for each well. Chemical compounds to be tested, CA, CAHA, FA and FAHA were added to the multiwell immediately after the cell suspension was plated, in increasing concentrations of 10µM, 50µM and 100µM. Wells only with cell suspension and DMSO were used as negative controls. After a 6 hours incubation period, 20µL of the anti-body mix (detailed below) was added to each well. Cell suspension was incubated for 30min at 4^{o}C in the dark. Subsets of T cells and B cells were determined by flow cytometry.

The above process was repeated a second time. Cell suspension was cultured in 12h and 24h incubation periods. Only FA, FAHA and CAHA compounds were added in increasing concentrations of 50µM, 100µM, 150µM and 200µM.

3.6.2 Human colon adenocarciona cell lines

To study the cytotoxicity of the synthesized compounds, in vitro studies were performed in two human colon cancer cell lines, namely C2BBe1 and WiDr.

C2BBe1 (brush border expressing) cell line was cloned from the Caco-2 cell line. The clone was selected on the basis of morphological homogeneity and exclusive apical villin localization. C2BBe1 cells form a polarized monolayer with an apical brush border (BB) morphologically comparable to that of the human colon. Isolated BB contained the microvillar proteins villin, fimbrin, sucrase-isomaltase, BB myosin-1 and the terminal web proteins fodrin and myosin II. These cells express substantial levels of BB myosin I similar to that of the human enterocyte [188].

WIDr cell line has been shown by DNA fingerprinting to be a derivative of HT-29, although deposited with the ATCC as a colon adenocarcinoma line. WIDr cells are negative for Colon Antigen 3 expression, are positive for keratin by immunoperoxidase staining. The cells expressed p53 antigen (the p53 produced has a G -> A mutation resulting in Arg -> His at position 273). Growth of WiDr cells is inhibited by TNF-α. Inhibitors of dihydrofolate reductase are highly cytotoxic to WiDr cells [189].

Cells were cultured in an adherent way according to American Type Culture Collections' instructions. Both cell lines were cultured in *Dulbecco's Modified Eagle's Medium* (DMEM, Sigma-Aldrich) and supplemented with 5% foetal bovine serum (FBS), 1% antibiotic and 0.25mM sodium pyruvate for DMEM. Cells were maintained at 37 ºC in a humidified atmosphere with 95% air and 5% CO_2.

3.7 Clonogenic assay

Clonogenic assay was used to study the effectiveness of CA, CAHA, FA and FAHA in cell survival and proliferation. One of the main features of cell death by apoptosis is the redistribution of fosfatildilserine, that in apoptotic cells binds to Annexin V (AV). Propidium Iodide (PI), which does not permeate viable cells, binds to DNA intercalating between the bases in late apoptotic and necrotic cells. Apoptosis is determined by flow cytometry by quantitation of the levels of phosfatidilserine in the outer membrane of apoptotic cells. Simultaneous use of AV and PI allows to distinguish between apoptosis and necrosis.

The above process using PBMCs was repeated. The chemical compounds CAHA, FA and FAHA were added to cell suspensions in concentrations of 200μM for CAHA and 150μM for FA and FAHA. Cell suspension was incubated for 24h. After 24h multiwell plates were centrifuged at 1500rpm for 5min and RPMI medium was carefully removed and replaced by fresh one. Cell cultures incubated for 4 days. After 4 days cell cultures were ressuspended and centrifuged at 2500rpm for 5min. Supernatant was discarded. 100μL of 1x binding buffer (eBioscience) was added, followed by 2.5μL of Annexin-V (BD Bioscience), 1μL PI (Sigma Aldrich). Cell mixture

was incubated for 15min at RT. Then 150µL of 1x binding buffer was added. Samples were analyzed by flow cytometry.

3.8 Cell proliferation assay

In order to evaluate cell proliferation in compound-treated and untreated cells, sulforhodamine B (SRB) assay was used. Cells were seeded in 48-well plates at a concentration of 50,000 cells/mL. After 24 hours, cells were treated with the vehicle of administration, DMSO, or with increasing concentrations (10µM to 200µM) of CAHA for 24 hours. After the incubation period, SRB assay was performed.

SRB is a colorimetric assay that consists in the electrostatic binding of the sulforhodamine B to the aminoacids, and represents a measure of cell proliferation based on the total protein content. For that, cells were washed two times with PBS and fixed with a freezing solution of 1% acetic acid in methanol, at 4ºC. After one hour of incubation, cells were allowed to air-dry. Then 100µL per well of SRB 0.4% (in 1% acetic acid solution) were added and the plate incubated in the dark at room temperature, for 1 hour. Excess dye was removed and the plates allowed to dry. To solubilize the protein bound-dye, 10mM Tris base solution (pH10) was added to each well. Absorbance was measured at 540nm using a reference filter at 690nm. The cytotoxicity was expressed as percentage of cell proliferation in relation to the control experiment, namely, cultures treated with the vehicle of administration. Each experiment was performed in triplicate and repeated in at least two independent experiments.

3.9 Enumeration of T and B cell subsets

In order to differentiate cell subsets, peripheral blood samples were labeled with surface anti-human monoclonal antibodies (mAbs). T cells were labeled with anti-CD4 FITC (clone: OKT4) or anti-CD4 PE (clone: OKT4), anti CD8 APC (clone: SK1), anti-CD25 PerCP-Cy5.5 (clone: M-A251) and anti-CD69 PE (clone: FN50) or anti-CD69 FITC (clone: FN50). B cells were labeled with anti-CD19 PerCP-Cy5.5 (clone: HIB19), anti-CD69 FITC (clone: FN50), anti-CD80 APC (clone: 2D10), anti-CD1d PE (clone: 1B1). All mAbs were purchased from BioLegend (San Diego, CA, USA), BD Bioscience (San Jose, CA, USA) and eBioscience (Frankfurt, Germany).

PBMCs were incubated with 30µL of mAbs mix (diluted 1.5:100 in 1x PBS).

3.10 Flow cytometry analysis

The analysis was performed using a six-parameter, four-color FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 15-nW argon laser. For each

assay, events were collected using Cell Quest software (Becton Dickinson) and analyzed using FlowJo X 10.0.7r2.

3.11 Flow cytometry evaluation of T and B cell subsets

Using Flow Cytometry B and T lymphocytes subsets could be evaluated by the differential surface expression of some markers: T helper cells ($CD4^+$), cytotoxic T cells ($CD8^+$), T effector cells ($CD4^+CD25^+$ and $CD8^+CD25^+$, which include regulatory Treg cells) and activated T cells ($CD4^+CD69^+$ and $CD8^+CD69^+$); B lymphocytes ($CD19^+$), B regulatory cells ($CD19^+CD1d^+$), late phase activated B cells ($CD19^+CD80^+$), early phase activation B cells ($CD19^+CD69^+$), (Fig.3 A-B).

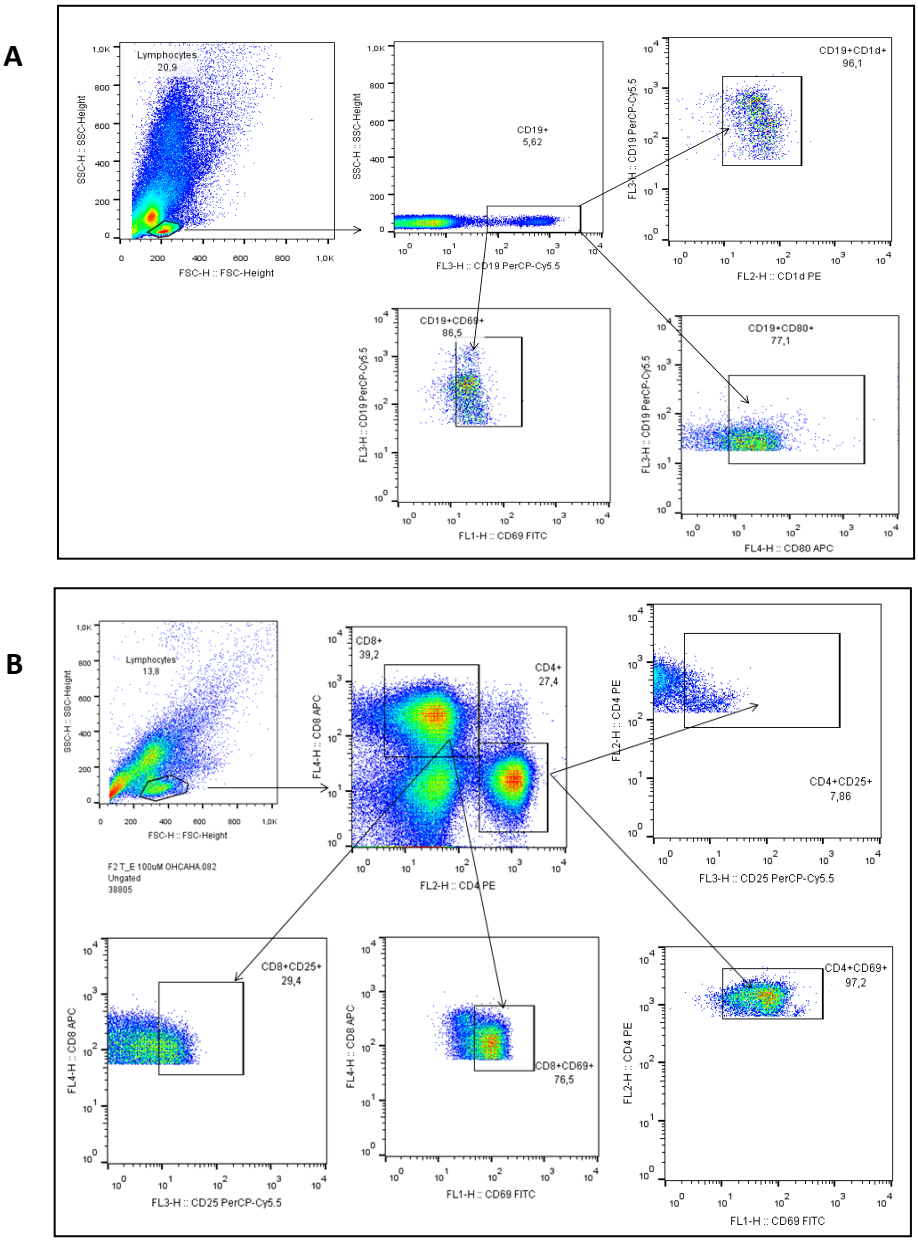

Fig.3 A) Representative dot plots of T lymphocytes and its subsets. B) Representative dot plots of B lymphocytes and its subsets.

3.12 Statistical analysis

Statistical tests were performed using GraphPad Prism Version 5.0 software (CA, USA). Since data did not follow a normal distribution the non-parametric Mann-Whitney and 2way ANOVA test was used to compare values between groups and time-points. Differences were considered significant for $p<0.05$.

Chapter 4

Results

4. Results

4.1 Clinical evaluation of DSS-induced colitis

Weight-loss was continually monitored throughout the entire study (Fig.4 A). At baseline the weight of Ncf1* and WT mice was comparable (WT=29.8±3.90g, Ncf1*=31.3±3.9g). Weight loss began on day 3 for the mice undergoing both DSS-induction protocols. At day 13, during the recovery period, all animals reached the minimum weight with Ncf1* mice presenting a significantly greater loss than WT. Weight recovery went all the way through day 30 for those mice in P1, with WT recovering the baseline weight whereas Ncf1* reached only up to 90% of the original weight. Those mice under P2 had a renewed weight loss starting on day 22 which went on until day 30, with Ncf1* mice presenting significantly greater loss than WT (Ncf1* reached 75% of baseline weight vs WT reaching 90% of baseline weight).

Stools consistency and the presence of blood in the feces are two further clinical signs of DSS-induced colitis [185]. Therefore, as with body-weight, mice were monitored for changes in stools throughout the study. During the 1st induction period (Day 0 through Day 7), both Ncf1* and WT groups had an accentuated decrease in stool. During the resting period both groups started to recover regarding the two clinical parameters. WT and Ncf1* mice submitted to P2 presented a new surge in anal bleeding as well softer stools which were significantly different for the clinical symptoms in those animals undergoing P1 (Fig.4 B and C).

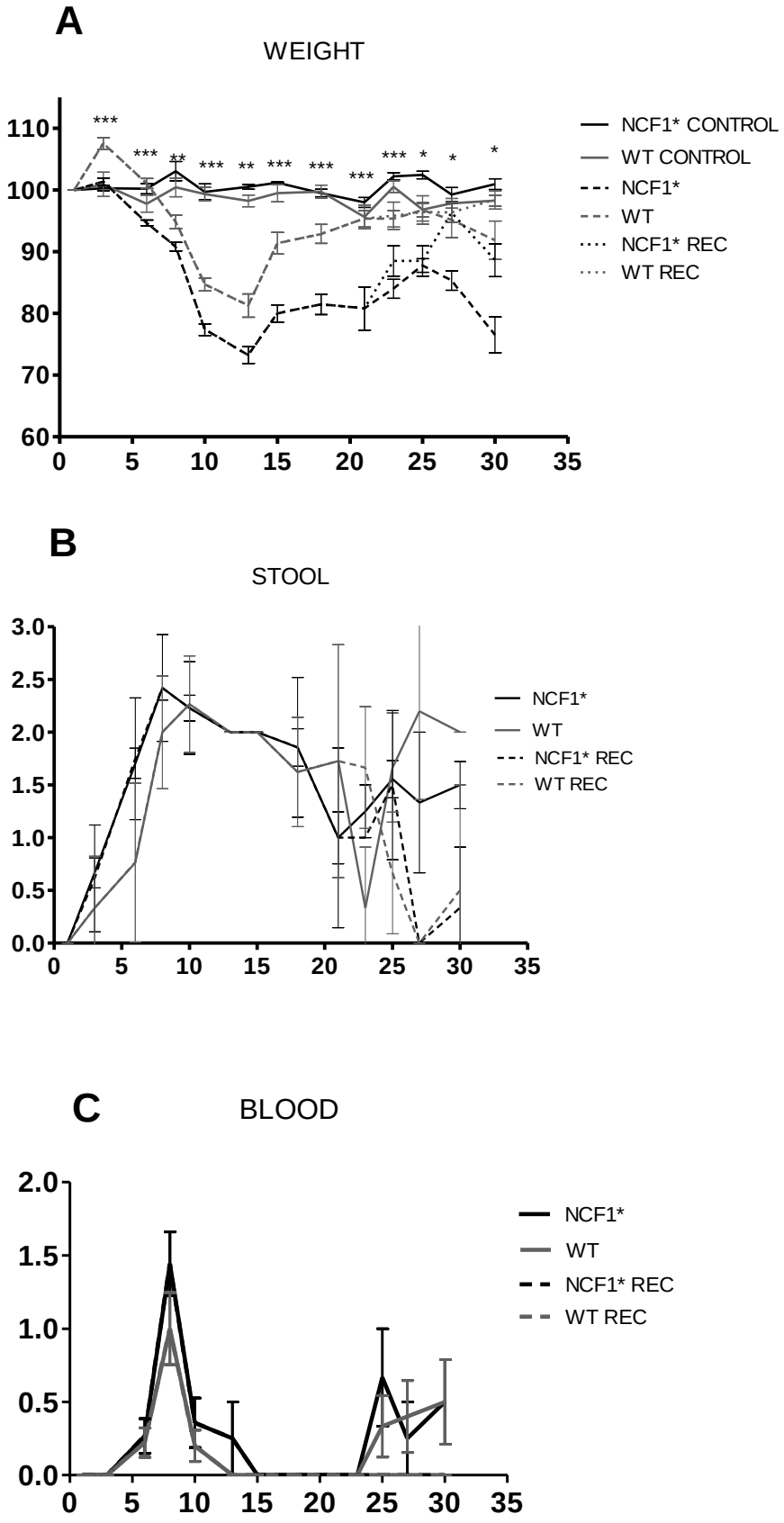

Fig.4 Ncf1* mice presented severer clinical scores than their WT counterparts. A) Mice weight distributions and analysis for all experiment. In experiment, average weight ± SE, (baseline weight: WT=29.8±3.90g, Ncf1=31.3±3.9g). B) Mice stool consistency scoring. C) Analysis of rectal blood presence ± SE. Weight, stool consistency and blood presence scorings are detailed in methods section. Asterisks indicate p<0.05, double asterisks p<0.001, triple asterisks p<0.0001. Mann-Whitney test was performed between each group, Ncf1* control/WT control; Ncf1*/WT; Ncf1* Rec/WT Rec; Ncf1*/Ncf1* Rec; WT/WT

Other parameters normally evaluated in this studies, are the colon's length and spleen's weight. The colon's shortening and the increase in spleen's weight as a consequence of a DSS induction are well known facts.

In both protocols the two mouse groups presented a significant reduction of colon's length after colitis induction (WT: D0 vs D22 p= 0.0159; Ncf1*: D0 vs D22 p= 0.0102). The length of the colon in both groups of P1 mice at day 30 remained equivalent to day 22, whereas WT mice following P2 presented a further significant reduction of the colon (p= 0.0025). Mice that were submitted to second DSS-cycle had significantly shorter colons than those which were allowed to rest after the first colitis induction (WT D30 P1vs P2 p= 0.0281; Ncf1* D30 P1 vs P2 p= 0.0229) (Fig.5 A).

Concerning the spleen's weight, at baseline Ncf1* mice presented a significant splenomegaly when compared to WT mice (p=0.0139). At day 22 there was a significant increase in spleen's weight in both groups when compared to baseline. Moreover, the Ncf1* mice had significantly heavier spleens than WT (p<0.0195). At day 30, regardless of protocol, both mice groups kept the high spleen weight as at day 22 (Fig.5 B).

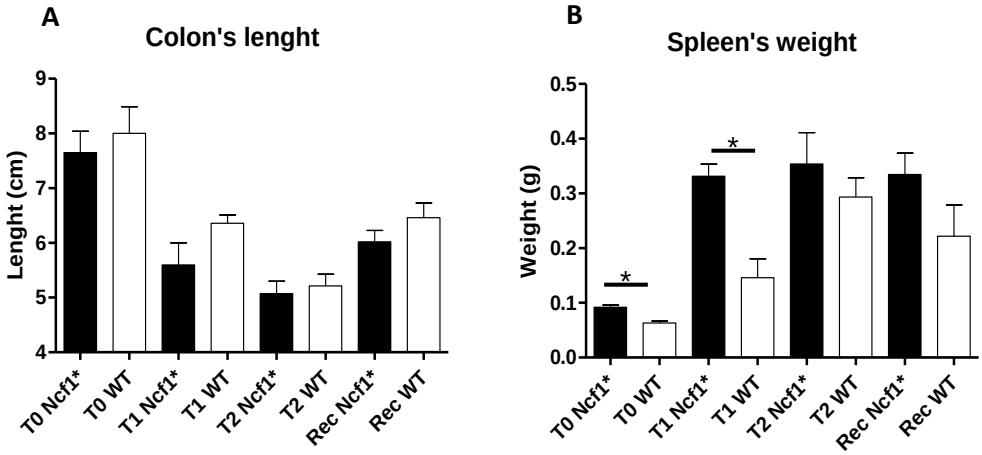

Fig.5 Colitis induction alters colons length and spleen weight in both animal groups. A) Colon's length in centimeters, for days 0, 22 and 30. B) Spleen's weight in grams for days 0, 22 and 30. Asterisks indicate p<0.05, Mann-Whitney test was performed between T0 Ncf1* and T0 WT; T1 Ncf1* and T1 WT; T2 Ncf1* and T2 WT and Rec Ncf1* and Rec WT.

4.2 Histopathological assessment of DSS-Induced Colitis

The assessment of colon histopathology score was done in untreated WT and Ncf1* animals, and after colitis induction at days 22 and 30 for P1 and at day 30 for P2, to evaluate dysplasia, epithelial morphology and inflammation, according to the described scoring criteria.

At baseline WT mice had well designed glands above *muscuralis mucosae*, whereas Ncf1* mice showed a reduced number of glandular tubules (Fig.6 and Table 1, Day 0).

On day 22 P1 WT mice maintained preserved epithelial morphology and villous projections plus low grade inflammatory reactive dysplasia. The Ncf1* group presented villous projections formed by compacted glands with less vascularization in the pedicle, plus glandular high grade dysplasia and invasive tubular adenocarcinoma foci (Fig.6, and Table 1, Day 22).

On day 30 the mice in P1 had reparative villous and glandular adaptation. WT mice maintained villous projections corresponding to half of the *mucosae* length, supported by tubular/glandular hyperplasia above the *muscularis mucosae*, with different sizes and segments of very small glands with low grade dysplasia foci. In Ncf1* mice colon there was a reduced number of glands under villous projections, although there persisted a morphology similar to day 22. In general Ncf1* mice showed high grade dysplasia (with anisocariosis with persistent nucleoli and visible mitosis) and foci of invasive tubular adenocarcinoma while WT colon presented basal glands' hyperplasia with low grade and high grade segments of dysplasia (Fig.6, Table 1, Day 30-P1).

On day 30 of P2, colonic sections showed persistent adaptation (Fig 6, Table 1, Day 30-P2): Ncf1* proximal and dystal colonic segments kept a glandular morphology with hypercromatic nuclei and mucosa-associated lymphoid tissue hyperplasia; and WT colon presented epithelial villous projections without inflammation. In distal segments, high grade dysplasia persisted in both groups. Additionally, Ncf1* colon developed invasive well-differentiated adenocarcinoma in segments where few reserve micro-glands were visible above *mucularis mucosae*, invading till the *muscular propria* (Fig.6, Table 1, Day 30-P2).

In general histopathological morphology descriptions had more inflammatory foci and glandular/epithelial alterations in the distal halves of colonic segments, summarized as follows:

The distal segment of the colon presented significantly higher inflammation score and higher grade dysplasia for Ncf1* mice ($p=0.259$ for inflammation and $p=0.0135$ for dysplasia, Fig 7 A-C). For those animals following P1 there was a better evolution of inflammation and dysplasia at day 30 in the WT group than in the Ncf1*.

Proximal colon inflammation score was significantly higher in Ncf1* mice regardless of induction protocol than in WT ($p=0.0357$ after the second induction) (Fig.7 B-D). Considering dysplasia, after the second induction, WT colon in both protocols presented significantly higher

scores than Ncf1* (p=0.0463). However, Ncf1* colon presented relevant incidence of invasive adenocarcinoma.

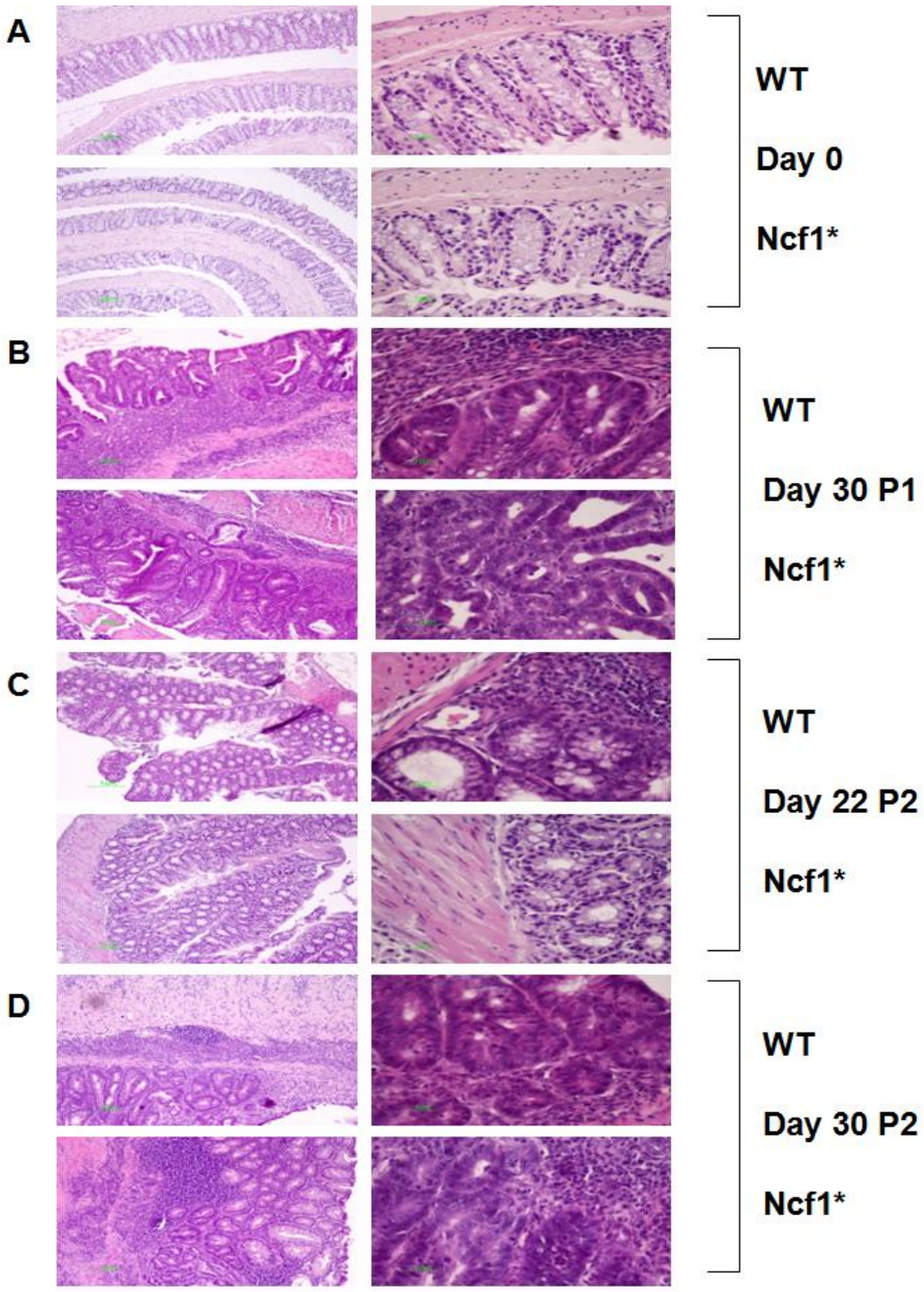

Fig.6 Colonic mucosa of control, P1 and P2 WT and Ncf1* mice groups. A) Day 0 of P1 and P2: glands are smaller, fewer and with small epithelial cells' nuclei in Ncf1* mice colon compared to WT. B) Day 30 of P1: WT mice colon with basal small glands hyperplasia (X) and high grade dysplasia; Ncf1* mice colon with superficial villous adaptation and invasive tubular adenocarcinoma. C) Day 22 of P2: Both WT and Ncf1* mice colon with superficial villous glandular hyperplasia with inflammation reactive atypia in WT mice colon; low grade dysplasia and mucinous cells hyperplasia in tubular glands of WT mice comparing with basal high grade dysplasia in Ncf1* mice. D) Day 30 of P2: Superficial villous mucosae and basal cell glandular persistence with high grade dysplasia in WT colon compared to superficial villous atrophy and invasive tubular (well differentiated) adenocarcinoma in Ncf1* colon. HE staining with 100x and 400x magnifications.

	WT	Ncf1*
Day 30 P1	• Basal glands hyperplasia • Segments of high grade dysplasia	• Superficial villous adaptation • Invasive tubular adenocarcinoma
Day 22 P2	• Villous superficial adaptation with reactive atypia • Tubular low grade dysplasia (mucinous cells hyperplasia)	• Villous superficial adaptation without reactive atypia • Tubular high grade dysplasia
Day 30 P2	• Villous superficial persistent tubular high grade dysplasia	• Villous superficial atrophy • Invasive tubular adenocarcinoma

Table 1- Comparison of the major histopathological findings.

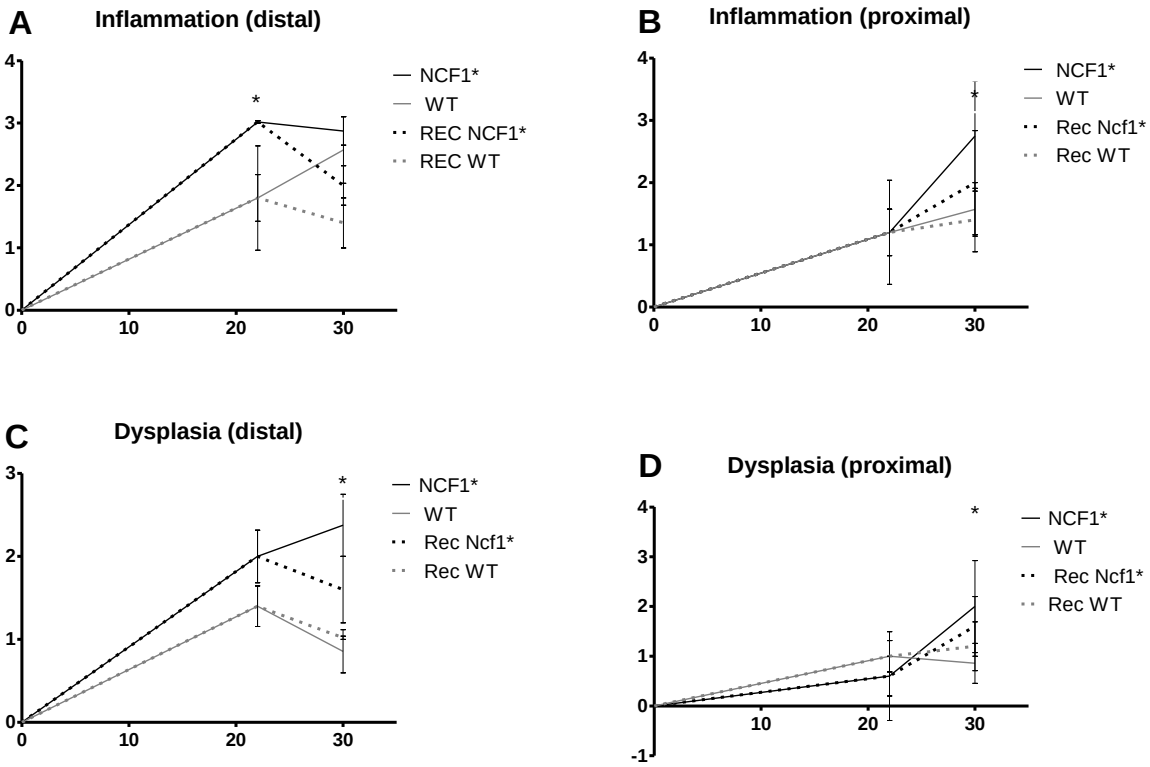

Fig.7 Histological evaluation of inflammation and dysplasia at distal and proximal segments of the colon for Ncf1* and WT mice. Asterisks indicate p<0.05, Mann-Whitney test between Ncf1* and WT and Ncf1* Rec and WT Rec.

4.3 Effects of caffeic-acid derivatives on colon-adenocarcinoma cell lines

Addition of CAHA to the medium presented inhibitory effects in C2BBe1 cell line in a dose-dependent manner (Fig.8 A). Although with no statistical significance inhibitory activity was stronger after 72h incubation when compared with 24h, that was notorious with 50µM-200µM. Similar effects were obtained when CAHA was added to WIDr cell culture, with the exception that 10µM did not produce inhibitory effect relatively to control (Fig.8 B). Higher concentrations of CAHA inhibited almost completely cell proliferation in WIDr, whereas C2BBe1 remains with 5% proliferation at 200µM.

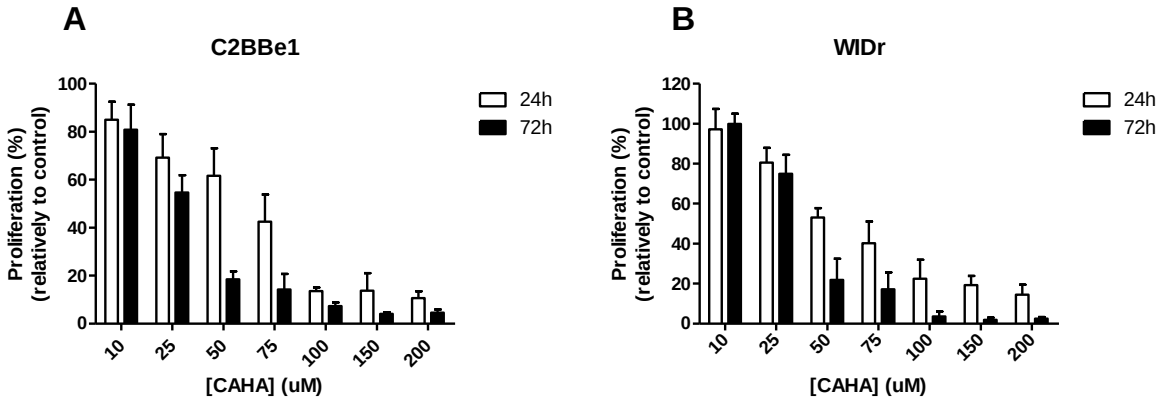

Fig.8 Effects of caffeic-acid derivatives on human colon-adenocarcinoma cell lines after 24h and 72h incubation periods. A) Effects of CAHA on C2BBe1 cell line. B) Effects of CAHA on WIDr cell line.

4.4 Effects of caffeic-acid derivatives on PBMCs after a 6h incubation period

The effects of CAHA on peripheral blood mononudear cells are shown in Fig. 9 A and B. The addition of CAHA to the medium increased $CD4^+CD25^+$ and $CD19^+$ cell subpopulations in a dose-dependent manner, with 100µM increasing proliferation 1.84 and 1.57-fold respectively, relatively to the control. When added in concentrations of 50µM and 100µM had a stimulatory effect on T helper cells ($CD4^+$) when compared to the control. Concentrations of 10µM-100µM had inhibitory effect on $CD19^+CD80^+$ and inhibited in a dose-dependent manner $CD19^+$ subpopulation, with 100µM decreasing 1.17-fold compared to the control. Cytotoxic T cells and $CD8^+CD25^+$ cells increased only with 10µM-50µM and 10µM respectively, although without reaching statistical significance.

Addition of caffeic acid increased cell percentage of $CD4^+$ cells with statistical significance between 10µM and 100µM ($p=0.049$), 100µM increased 1.42-fold comparatively to the control. CA had stimulatory effect on $CD4^+CD25^+$ cells (100µM increased 1.24-fold compared to the control), $CD8^+$ cells and $CD19^+$ cells (Fig.10 A-B). Similar results to CAHA were

seen in $CD19^+CD1d^+$, $CD19^+CD80^+$ and $CD8^+CD25^+$ cell subpopulations, where in $CD8^+CD25^+$ 100µM increased 1.43-fold comparing to the control.

Results relative to PBMCs treated with FA (Fig.11 A-B) did not show any alterations in $CD4^+$ and $CD8^+$ cell subpopulations, when comparing them to the controls. However effector T cells percentage increased in a dose-dependent manner, but without any statistical significance. In $CD4^+/25^+$ 100µM increased 2.26-fold and in $CD8^+/CD25^+$ 100µM increased 2.07-fold relatively to the control (Fig.11 A). In the case of $CD19^+$ and $CD19^+CD80^+$ cells only 10µM increased cell percentage, 1.23-fold and 1.62-fold respectively, when compared to the control, whereas other concentrations had inhibitory effects. B regulatory cells did not suffer any changes relatively to the control, with the exception of 10µM that had a small inhibitory effect, although with no statistical significance (Fig.11 B).

When added to the medium FAHA only increased cell percentage of T effector subpopulations. In $CD4^+CD25^+$ cells, 10µM had a 22-fold increase comparing to all other concentrations and in $CD8^+CD25^+$ subpopulation 10µM-100µM had a 1.49, 1.35 and 1.55-fold increase respectively, although with no statistical significance (Fig.12 A). T helper and T cytotoxic subpopulations maintained equal cell percentage after the treatment relatively to the control, except with 10µM, that had a small inhibitory effect on cell proliferation. In B subpopulations, $CD19^+$ and $CD19^+CD1d^+$ did not suffer any changes, contrary to $CD19^+CD80^+$ where FAHA treatment had inhibitory effect on cell proliferation, without statistical significance (Fig.12 B).

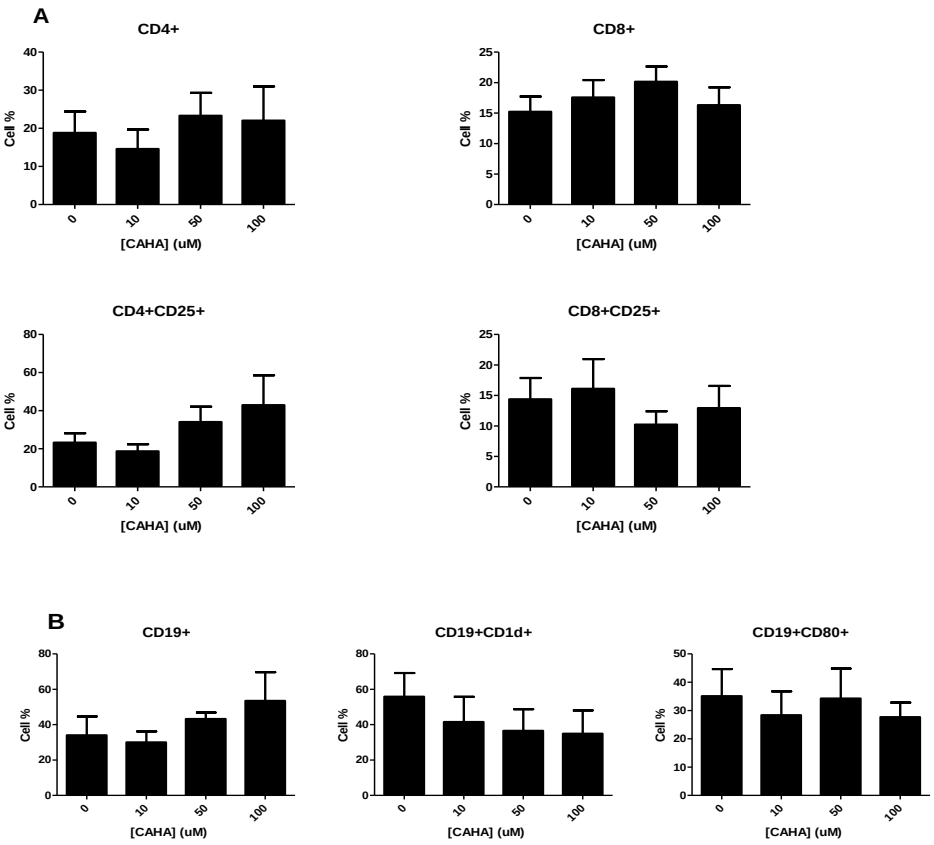

Fig.9 PBMCs incubated for 6h period with CAHA in increasing concentrations of 0µM-100µM. A) T lymphocytes cell percentage subsets. B) B lymphocytes cell percentage subsets.

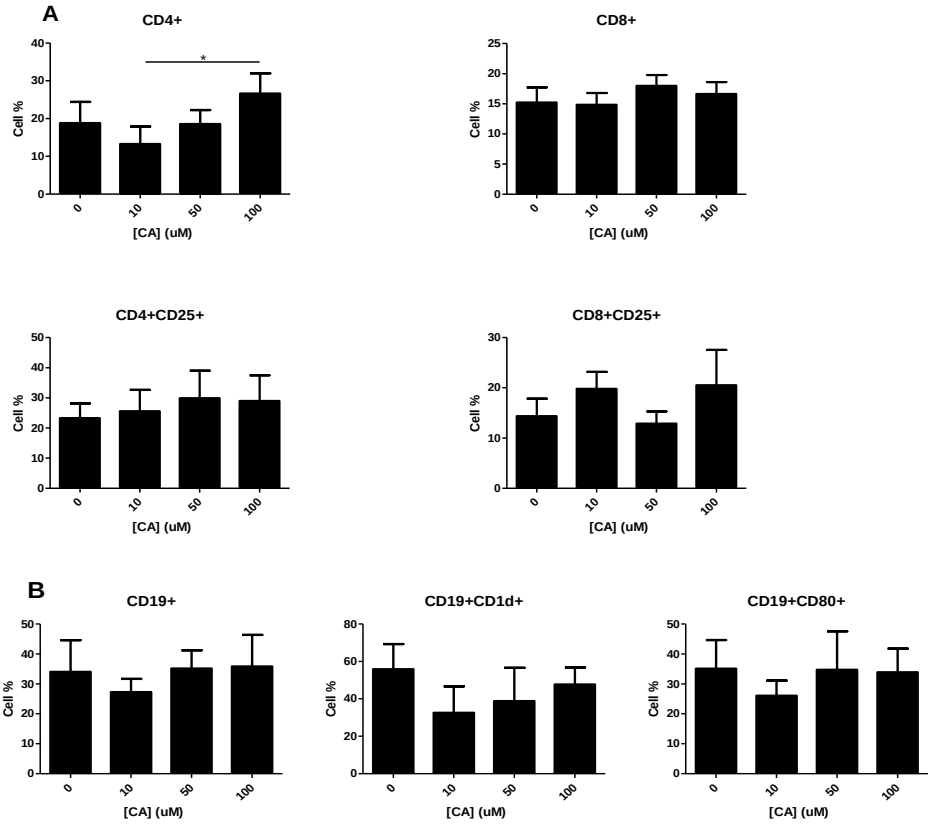

Fig.10 PBMCs incubated for 6h period with CA in increasing concentrations of 0µM-100µM. A) T lymphocytes cell percentage subsets. B) B lymphocytes cell percentage subsets. *p<0.05. Mann Whitney test.

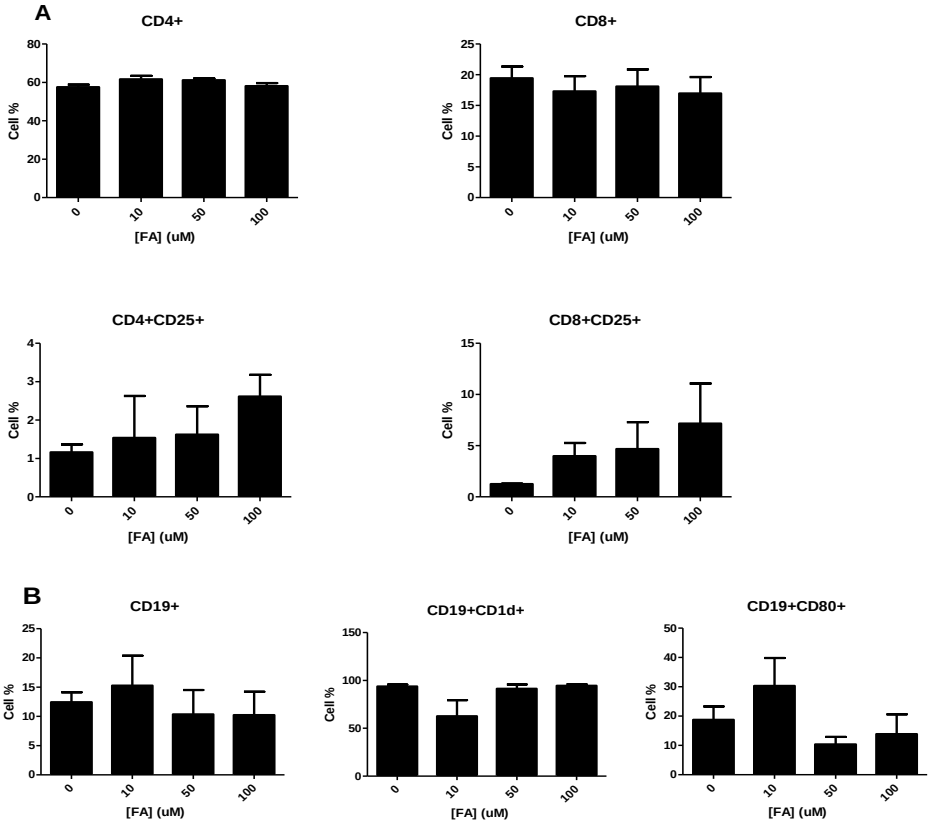

Fig.11 PBMCs incubated for 6h period with FA in increasing concentrations of 0µM-100µM. A) T lymphocytes cell percentage subsets. B) B lymphocytes cell percentage subsets.

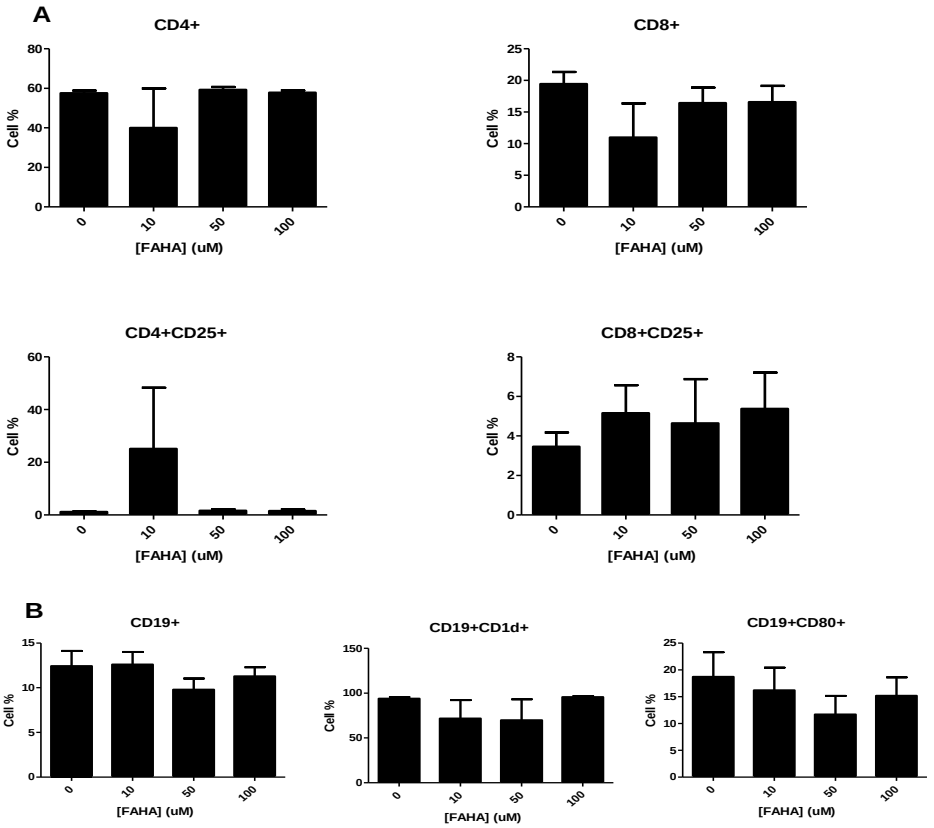

Fig.12 PBMCs incubated for 6h period with FAHA in increasing concentrations of 0µM-100µM. A) T lymphocytes cell percentage subsets. B) B lymphocytes cell percentage subsets.

4.5 Effects of caffeic acid derivatives on PBMCs after a 12h and 24h incubation period

Lymphocytes are generally slow response cells, so only 6h of incubation with the caffeic acid derivatives may not be enough to achieve stimulatory/inhibitory results, hence we decided to study the long-term effects of the compounds with 12h and 24h incubation periods.

When cells were treated with 0-200µM of CAHA for 12h and 24h a small inhibitory effect was seen in T helper cells in a dose-dependent manner (with 200µM cells had a 2.2-fold decrease, relatively to control) (Fig.13 A). Cytotoxic T cells did not present any changes between concentrations and incubation periods (Fig.13 B), and the same happened to activated T cells ($CD4^+CD69^+$ and $CD8^+CD69^+$). However expression of CD69 in $CD69^+$ cells increased in a dose-dependent manner in both $CD4^+CD69^+$ and $CD8^+CD69^+$ cell subpopulations, being 24h of incubation more favorable to the expression of CD69 protein, with 200µM increasing 5.14-fold and 4.20-fold the expression of CD69 in $CD4^+CD69^+$ and $CD8^+CD69^+$ cell subpopulations respectively, although with no statistical significance. Whereas the expression of CD25 in $CD25^+$ cells did not show any relevant alterations, although in $CD8^+CD25^+$ CD25 protein expression had a 1.72-fold increase relatively to the control. Effector T cells increased with the concentration of CAHA, $CD4^+CD25^+$ presented better results with the 24h incubation, with statistical significance between 200µM ($p<0.05$), increasing 2.95-fold. The contrary was seen in $CD8^+CD25^+$, with 12h incubation having significant results for 50µM, 100µM, 150µM and 200µM ($p<0.01$ to all concentrations).

Results from activated ($CD19^+CD69^+$) and regulatory ($CD19^+CD1d^+$) B cells presented statistical significance between 50µM, $p<0.001$ for both (Fig.13 C). Despite the results there was a tendency to 200µM with a 24h incubation to promote cell proliferation ($CD19^+$ with 200µM had a 2.80-fold increase relatively to control). Although with no statistical relevance 200µM stimulated the expression of CD80, CD69 and $CD1d^+$ protein, suffering a 3.61-fold, 4.43-fold and 6.27-fold increase relatively to untreated cells.

Addition of FA to the medium increased cell proliferation of $CD4^+$ and $CD8^+$ subpopulations after 24h comparing to 12h incubation. T helper cells presented statistical significant results between 50µM-100µM $p<0.01$ and 150µM-200µM $p<0.001$, cell proliferation increased an average of 2.96-fold from 12h to 24h incubation (Fig.14 A), as for cytotoxic T cells the statistical significant results were only between 200M $p<0.05$ (Fig.14 B). Treatment with FA did not alter activated T cell subpopulations comparing with controls, neither the expression of CD69 in $CD69^+$ cells, although without statistical relevance after 24h incubation, 50µM increased 2.53-fold and 2.39-fold the expression of CD69 protein in $CD4^+CD69^+$ and $CD8^+CD69^+$ respectively. Effector T cell subpopulation increased after 12h incubation, being 100µM-200µM more favorable to cell proliferation.

FA increased an average of 8.52-fold in $CD19^+$ subpopulation after 24h incubation comparing to 12h incubation, however there with no statistical significance between concentrations. In B lymphocytes subsets, only regulatory B cells presented statistical significant results between 50µM $p<0.05$ (Fig.14 C). Overall, concerning cell proliferation, FA results were better after 24h incubation period.

The addition of FAHA to PBMCs culture lead to a significant increase -after 24h incubation- of T helper (an average of 3.01-fold increase) (Fig.14 A) and T cytotoxic cells (an average of 3.70-fold increase) (Fig.15 B) subpopulation for 50µM-200µM concentrations (for $CD4^+$ subset, 50µM $p<0.001$ and 100µM-200µM $p<0.01$, as for $CD8^+$ 50µM-200µM $p<0.05$). Activated T cells did not present any alterations with the treatment and as previously seen with FA, effector T cells proliferation increased after 12h comparing to 24h incubation ($CD8^+CD25^+$ 50µM $p<0.05$). After 24h incubation cell percentage was maintained independently of the FAHA concentration.

In the results relatively to B lymphocytes subpopulations (Fig.15 C), activated B cells were the only subpopulation presenting alterations ($CD19^+CD80^+$) between 200µM $p<0.001$.

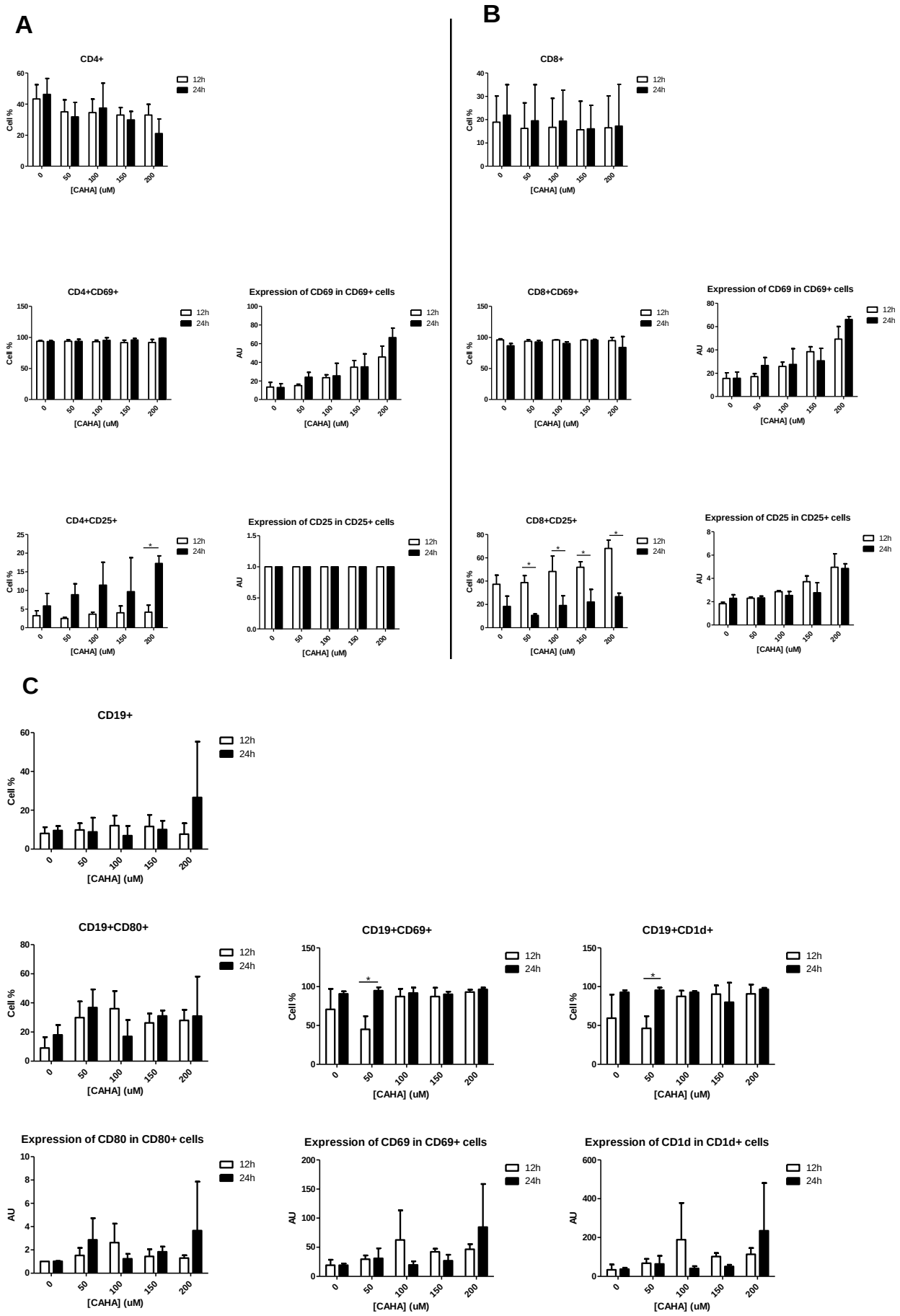

Fig.13 PBMCs incubated for 12h (white bars) and 24h (black bars) periods with CAHA in increasing concentrations of 0µM-200µM. A-B) T lymphocytes cell percentage subsets. C) B lymphocytes cell percentage subsets. Mann Whitney test and 2way ANOVA was performed between concentrations and between 12h-24h incubation periods, *p<0.05.

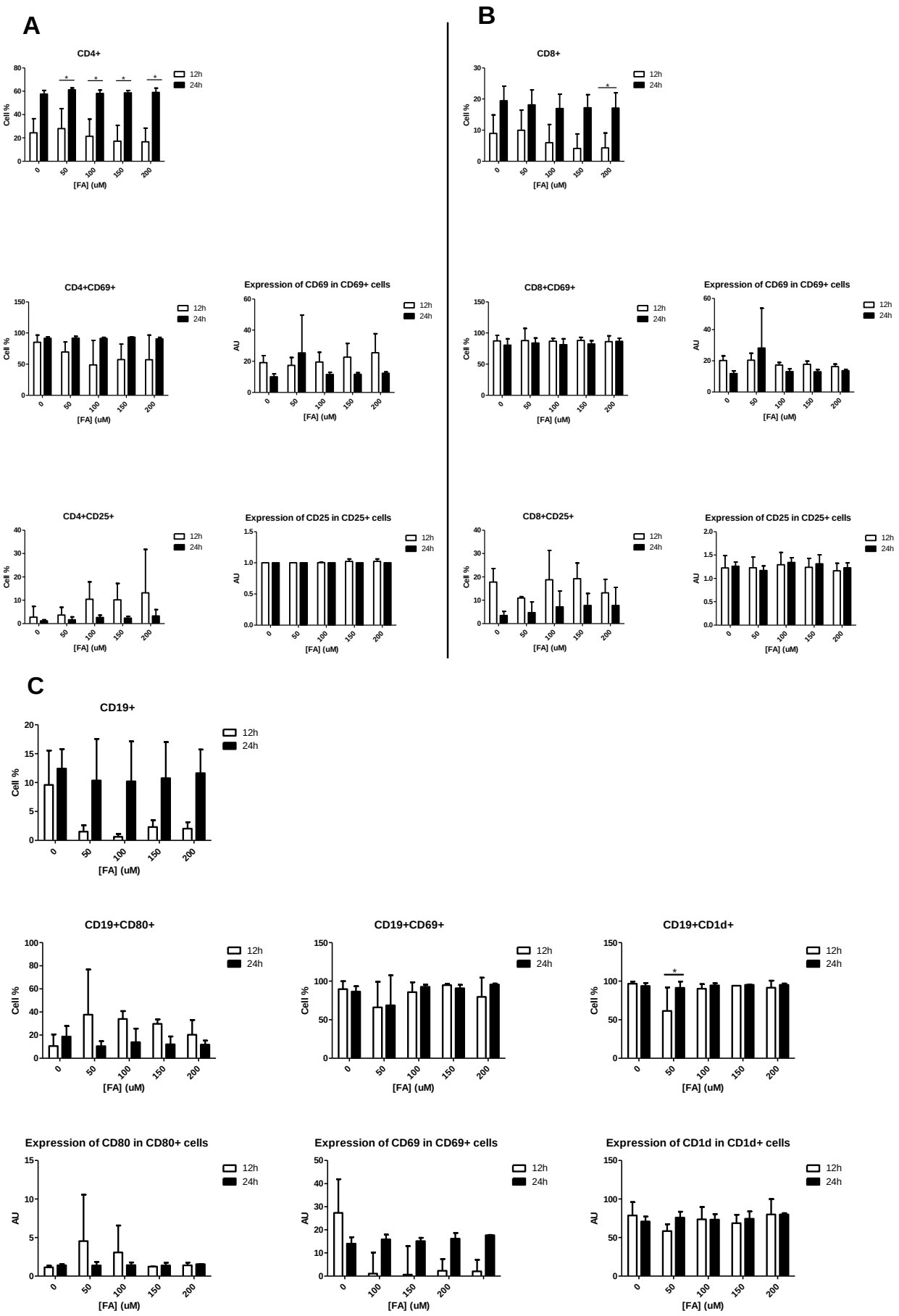

Fig.14 PBMCs incubated for 12h (white bars) and 24h (black bars) periods with FA in increasing concentrations of 0µM-200µM. A-B) T lymphocytes cell percentage subsets. C) B lymphocytes cell percentage subsets. Mann Whitney test and 2way ANOVA was performed between concentrations and between 12h-24h incubation periods, *p<0.05.

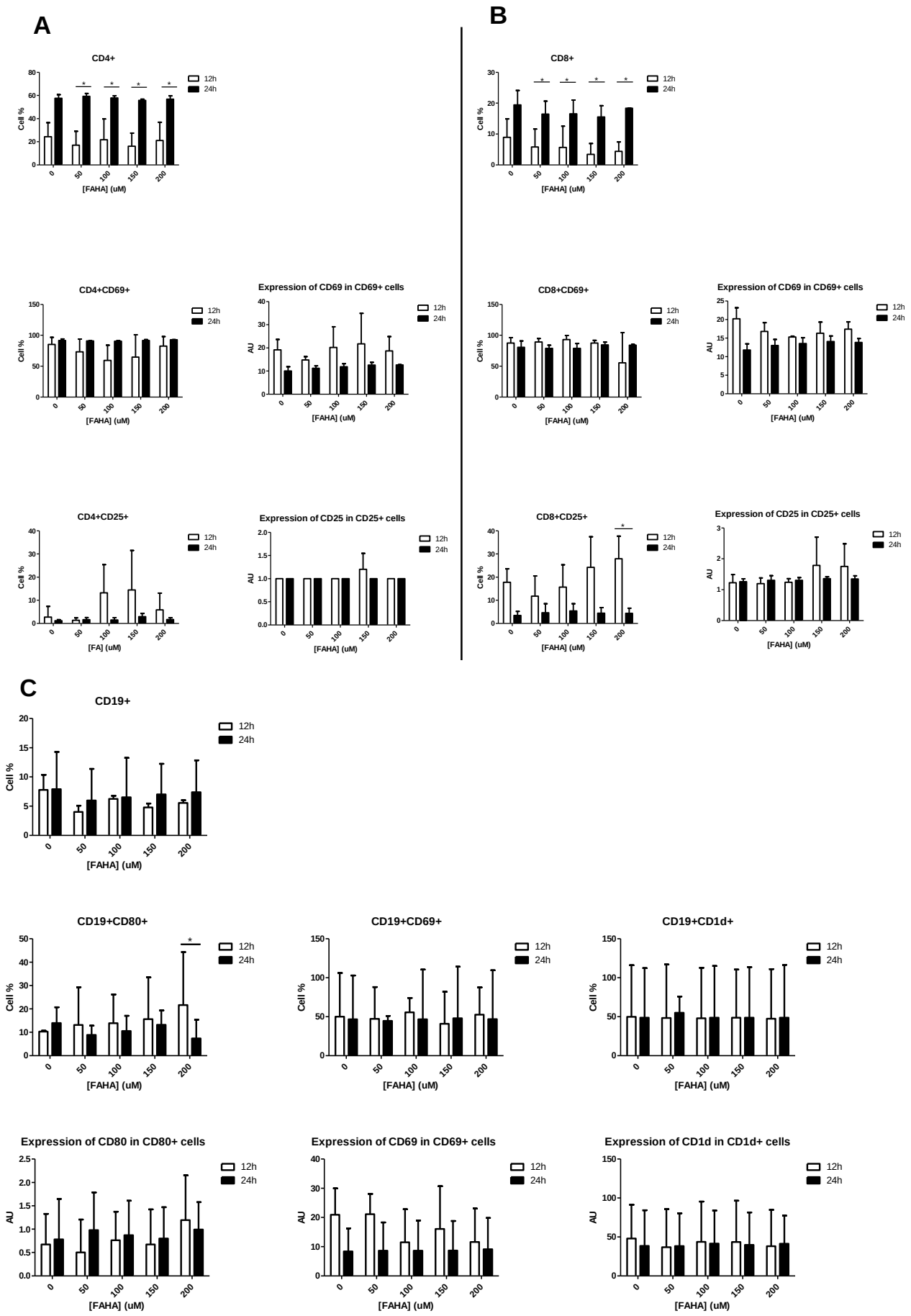

Fig.15 PBMCs incubated for 12h (white bars) and 24h (bars) periods with FAHA in increasing concentrations of 0μM-200μM. A-B) T lymphocytes cell percentage subsets. C) B lymphocytes cell percentage subsets. Mann Whitney test and 2way ANOVA was performed between concentrations and between 12h-24h incubation periods, *p<0.05.

4.6 Effects of caffeic acid derivatives on survival and proliferation of PBMCs

The incorporation of AV or PI allowed an assessment of the effectiveness of the treatments (CAHA, FA and FAHA) which could be evaluated by cell survival and proliferation (Fig.16).

Examination of cell survival in the presence of 200µM of CAHA indicated that almost 80% of the cells remain viable after 4d of incubation and still proliferate when comparing to early apoptotic cells (p=0.0002), late apoptotic cells (p=0.0002) and necrotic cells (p=0.0002). Early apoptotic cells and late apoptotic cells percentage decreased after the treatment when compared to the controls, p=0.055 and p=0.0002 respectively. Necrotic cells when compared to early apoptotic cells and late apoptotic cells also presented relevant differences (p=0.0003, p=0.0002 respectively) (Fig.17 A).

Results of cell survival and proliferation with 150µM FA were similar to CAHA. The majority of the cells continued viable compared with early apoptotic, late apoptotic and necrotic cells (p=0.0002 to all). Early apoptotic cell percentage was higher than late apoptotic cells (p=0.0043) and the same to necrotic cells compared to late apoptotic cells (p=0.0002) (Fig.17 B).

Treatment with 150µM FAHA contrary to previous results inhibited cell proliferation comparing to the control, although not significant. Still cell survival was higher when compared with early apoptotic (p=0.0087), late apoptotic (p= 0.0022) and necrotic cells (p=0.002). Late apoptotic cells presented the lowest percentage when comparing with early apoptotic and necrotic cells (p=0.0022 to both) (Fig.17 C)

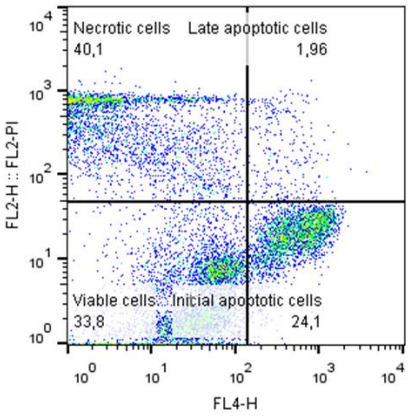

Fig.16 Representative dot plots of viable cells (PI^-AV^-), early apoptotic cells (PI^-AV^+), late apoptotic cells (PI^+AV^+) and necrotic cells (PI^+AV^-).

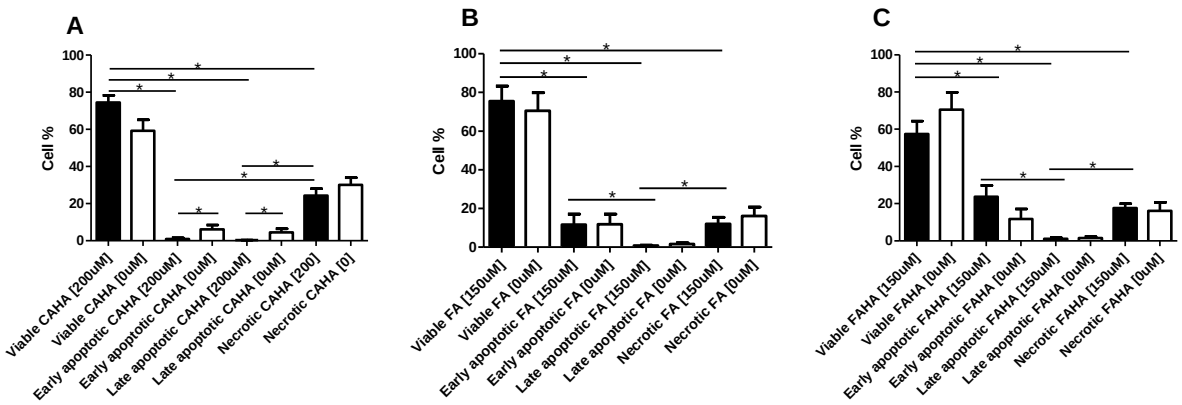

Fig.17 Clonogenic assay-effectiveness of the treatment in cell survival and proliferation. A) CAHA 200M B) FA 150M. C) FAHA 150M. Treated cells - black bars, controls - white bars. Mann Whitney test was *p<0.05.

Chapter 5

Discussion/Conclusion

5. Discussion and conclusion

Inflammatory Bowel Disease is a chronic relapsing idiopathic immunological disorder of the gastrointestinal tract, leading to long-term and sometimes permanent impairment of gastro-intestinal structure [1, 2]. IBD encompasses Crohn's Disease and Ulcerative Colitis. Evidence suggests that IBD arises from an inappropriate inflammatory immune response to intestinal microbes in a genetically susceptible host. Even though UC and CD have many clinical and pathological characteristics in common, key features separate both diseases [2-9]. Within the category of primary immunodeficiency IBD depends on mutations in the NADPH oxidase complex, responsible for the production of ROS [9, 56, 62, 72]. In particular, a point mutation in the Ncf1 gene in B10.Q mice, as in humans, impairs the production of ROS, rendering them to increased susceptibility to bacterial infections [190], and to the development of severe chronic autoimmune disorders [191, 192]. The hyper inflammatory responses are directly associated to the inability of producing ROS, since the administration of oxidants leads to permanent recovery in treated animals [191, 192]. It is already known that during the acute phase of colitis ROS are essential to inflammation's resolution [185], however studies focusing on the role of ROS-deficiency and how chronic colon-inflammation evolves to carcinogenesis are still missing. Furthermore, there is urgent need for effective therapies which promote simultaneously cancer cell death and immune system activation.

Hence the aim was to study how the lack of ROS influenced chronic DSS-induced colitis and disease evolution to colon-adenocarcinoma in Ncf1-deficient mice. Moreover we proposed to test the therapeutic and immune-stimulatory effectiveness of caffeic acid-derivatives in human PBMCs and human colon adenocarciona cell lines.

The DSS-colitis model is commonly used in a variety of mouse strains because it is simple to administrate, acts rapid, its duration and severity of inflammation are controllable and its ability to induce disease in a great variety of mice strains [193]. Depending on the number of DSS-cycles or concentration, the disease can evolve from acute to chronic inflammation and even further to dysplasia. Furthermore, dysplasia that resembles the clinical course of human UC occurs frequently in the chronic phase of DSS-induced colitis [194]. Previous studies have shown that Ncf1* mice treated with several DSS-cycles intercalated by a resting period on normal water develop severer colitis, contrary to studies focused on a single DSS-round of colitis induction [185]. Previous studies have demonstrated that different mice strains are highly susceptible to colon carcinogenesis from the exposure to DSS, as IL-$10^{-/-}$, $Smad3^{-/-}$ TGF-β, $APC^{+/Min}$ [132, 134, 136, 137, 139, 195, 196]. The administration of a carcinogen alongside with DSS is often preferred, since it speeds up all the process [197-199].

In the present work Ncf1* mice with a single 7 days exposure to 3% DSS in drinking water followed by a 14 days resting period developed colonic high grade dysplasia when compared with low grade dysplasia found in the colon of WT mice. Furthermore after the 3 weeks resting period, we observed lower inflammatory reparation in Ncf1* together with high grade dysplasia and invasive adenocarcinoma, while in WT high grade dysplasia was also prominent without malignant invasion. When low-grade dysplasia is detected there is a 9-fold risk of developing cancer, whereas high-grade dysplasia carries a 43% risk of malignancy [200, 201]. A review by Triantafillidis et al. hypothesizes that in mechanisms concerning carcinogenesis, inflammation results in neoplastic transformation, by enhancing epithelial cell turnover in the colonic mucosa. Biopsies of areas with active inflammation from UC patients demonstrated higher rates of mitosis and apoptosis then seen in healthy control individuals [122]. Sturlan Oberhuber et al. showed that IL-10 $^{-/-}$ mice spontaneously develop colitis which leads to high grade dysplasia, which indicates that with compromised immune response the propensity for dysplasia increases [131, 132].

Clinical signs of acute DSS-induced colitis were possible to be observed after the first DSS-cycle, however clinical signs of the chronic phase of the disease do not usually reflect the severity of inflammation or the histological features found in the bowel [193]. DSS toxicity to the colonic epithelial cells causes defects in the epithelial barrier integrity, increasing the colonic mucosal permeability, to allow penetration of large molecules like DSS and the entry of luminal antigens in the mucosa resulting in overwhelming inflammatory response. Decreased stool consistency and diarrhea are responsible for the marked weight loss seen in the Ncf1* mice. The observed shortening of the bowel was more intense in Ncf1* mice, it is caused by the mucosa destruction. DSS treatment shortens the colon by contracture of the smooth muscle tissue [202-204].

This results point-out this animal model as a novel tool to study chronic colitis-induced carcinogenesis. Several studies report either a carcinogenic path dependent on longer periods/multiple cycles of DSS-induction, or use carcinogenic coadjutants to reduce the exposure periods. Cooper HS. *et al* used four-cycles of DSS induction in mice to correlate disease-length with the development of colitis-associated dysplasia and adenocarcinoma [123], modelling what is thought to happen in humans. In other animal models dysplasic lesions take a long time to develop [123], hence disease development is not only treatment-dependent, inherent to the strain there is a susceptibility level that may vary [205]. This contrasts with our model that uses just two induction cycles leading to adenocarcinoma.

Our study reveals some, yet unaddressed, particularities of the development of colon adenocarcinoma. In the histological analysis of the colon from our model, we followed the

2010 WHO classification of tumors of the digestive system [186] applying the human nomenclature for low grade dysplasia and high grade dysplasia/ intraepithelial neoplasia. As already clearly demonstrated stimulus-dependent inflammation is the currently recognized path for the development of pre-neoplastic lesions either in non-polypoid or in polypoid mucosa. In our model we could identify epithelial polypoid lesions/ adenomas (conventional tubular, villous and tubulo-villous), traditional serrated adenomas; senile serrated adenoma/ polyp without or with dysplasia and hamartoma-associated dysplasia.

Only conventional tubular, tubulo-villous and villous alterations of the flat mucosa superimposed to inflammation were observed without the formation of senile adenomas. Considering our previous observations in a model of acute DSS-colitis in the same mice [185] and the current chronic model, we can suggest that the inflammation process in Ncf1* colon is dependent of a spectrum from acute to adaptive presence of inflammatory cells.

ROS-influence in the studied carcinogenesis was underlined by the morphological alterations that were more prominent in Ncf1* mice, whereas the presence of ROS allowed mucosal adaptation in WT mice. As previously shown, iNOS is continuously overexpressed in the colon of Ncf1* mice after DSS-induction, but only transiently in WT counterparts [185]. Since high iNOS levels have been implicated in the progression from UC to colon carcinogenesis [140-145], we propose that the chronic process keeps iNOS overexpression in Ncf1* colon, which promotes the high grade dysplasia and adenocarcinoma formation.

The most relevant finding of the present study is that villous/superficial papillary adaptation of the flat mucosa was not relevant to adenocarcinoma morphology, while basal glands, the hallmark of WT response, were mostly irrelevant in Ncf1* mice, which displayed installed dysplasia quickly evolving to invasive well-differentiated adenocarcinoma. The WHO nomenclature and other studies [186, 206] recognizes several patterns for colonic tumors: adenocarcinoma, mucinous adenocarcinoma, signet-ring cell carcinoma and undifferentiated carcinoma. Our model was only capable of inducing well differentiated tubular/glandular adenocarcinoma, with sporadic mucous cells' hyperplasia in foci of high grade dysplasia. These findings are important as they suggest that villous patterns in villous and tubulo-villous adenomas may not be relevant for carcinogenesis and may correspond to an epithelial adaptive modification, as observed in gastric peptic ulcers re-epithelization [207].

Colonic adenocarcinoma is associated to hereditary genetic traits as well as environmental factors and ageing [208]. In ageing individual atherosclerotic mucosal atrophy and diet may be associated with what pathologists classify in routine biopsies as chronic atrophic colitis, morphologically similar to the Ncf1* mice mucosa. As anti-oxidants-rich diets are becoming wide-spread among the ageing population in westernized countries, our data

stress the indispensability of carrying out studies on how chronic diet-induced reduction of ROS may be implicated in colon carcinogenesis in humans. Moreover, if glandular atrophy or reduced number of glands above muscularis mucosae becomes demonstrated as a risk factor or as a pre-neoplastic condition, it will be more appropriate to introduce the concept of intramucosal neoplasia instead of intraepithelial neoplasia, which is currently equivalent to high grade dysplasia and does not correlate with adenocarcinoma aggressive invasion as it was demonstrated in this study.

Caffeic-acid derivatives possess anti-inflammatory, anti-oxidant and anti-tumor properties already reviewed in literature [158-161]. Thus, the purpose of their use in the present study was to evaluate if they could be considered novel anticancer agents with a dual role, which kills cancer cells while promoting an activation of the adaptive immune system.

To assess the potential anticancer activity of the caffeic-acid derivatives, CAHA was incubated with C2BBe1 and WIDr human colon-cancer cell lines, where the results showed a markedly inhibition of cell proliferation in a dose-dependent manner. Therefore, our results corroborate similar conclusions already obtained in previous studies. Rao Desai and its team studied the antimutagenic and antitumorigenic activities of several synthesized caffeic-acid esters, were they significantly inhibited the growth of human colon HT-29 and HCT-116 cells at much lower concentration, when compared to CA [167]. CA notably inhibited HCT15 colon cancer cell proliferation in a dose-dependent manner in a study of CA-induced apoptosis mechanisms [169].

Our interest on these compounds was to determine whether they had low toxicity on human PBMCs, so we performed a clonogenic assay, hypothesizing that they would not be toxic to the adaptive immune system, while potentiating the activation of the immune cells. In a first phase assay all compounds enhanced cell proliferation of T helper, T cytotoxic and most importantly T effector cells (which include T regulatory cells). Based on the referred results, a second phase assay used concentrations were higher and longer incubation periods. CA was left out in this phase, due to previous results, where its effect on $CD4^+$, $CD4^+/CD25^+$ and $CD19^+/CD1b^+$ cell subpopulations presented inferior cell proliferation values, when compared to the other caffeic-acid derivatives. Due to CA structure, higher concentrations are needed to achieve the same results. Similar conclusions were already described in previous studies, where caffeic acid ester derivatives were more lipophilic than CA and thus easily facilitate their entry into the cell, requiring small amounts to exhibit their effective stimulatory activities [163]. When incubated during 12h and 24h, PBMCs reacted better to 24h incubation period

and as before, caffeic-acid derivatives enhanced effector T cells proliferation. Overall PBMCs proliferation increased more than after 6h incubation and with higher concentrations.

Corresponding to our expectations the used caffeic-acid derivatives showed to have very low cytotoxicity towards immune cells, since about 80% remain viable with all three compounds, contrary to very low percentages observed in early apoptotic, late apoptotic and necrotic cells, while potentiating the activation of the immune adaptive cells.

Enhanced effector T cell proliferation induced by the caffeic acid derivative compounds will potentiate the immune inflammatory response, through cytokine secretion and activation of other immune cells, such as T helper cells (Th1 and Th2) and cytotoxic T cells. Cytotoxic T cells are of extreme importance, because they are responsible for the recognition and elimination of cells that express antigens derived from intracellular bacteria and also mutated or embryonic proteins, generated by cells that have undergone malignant transformation. It is still uncertain the extent to which T $CD8^+$ cells are capable of controlling tumorigenesis development and progression, but is clear that deficiency in these cells increases the potential malignancy development [209]. Enhanced function of cytotoxic T cells allow robust antitumor responses in both animal models and human [210], although insufficient to fully protect from tumors.

The possible proliferative effect of the compounds on Treg cells proliferation would be important in tumor prevention, as they function to maintain immune homeostasis and limit the exacerbated immune response, they are effective in the prevention and down-regulation of inflammation of IBD in animal models. It is now hypothesized that Tregs might suppress inflammation induced by growth promoting immune system cells [211]. Although Treg cells are present in peripheral blood and intestine of IBD patients, active disease is characterized by contraction of the peripheral Treg cell pool and insufficient increase of Treg cells in the intestinal lesion [212]. Furthermore studies on Treg cells associated high levels of the same with a favorable prognosis in CRC [213].

Even though we shed a new light into the dual capacity of caffeic acid derivatives to promote both cancer cell death and activation of the adaptive immune system cells, further studies must be carried out, in order to assess their full capacity as novel drug candidates. Those studies should focus on their effects on the innate immune cells (dendritic cells, monocytes and NK cells). Additionally, *in vitro* co-cultures of caffeic-acid treated PBMCs with C2BBe1 and WIDr cell lines, would provide important data on whether the tumor death promoted by the compounds can be potentiated by the presence of activated immune cells. Another important issue, is to test the preventive action of these compounds in active IBD patients, mainly through their capacity to promote the expansion of the Treg pool. This could

be initially studied using the Ncf1*-model by administering these compounds in physiologically active doses prior to DSS-colitis induction.

Chapter 6

Supplements

Virchows Archiv

Colorectal Adenocarcinoma: Experimental model in oxidative burst Ncf1-mutant mice with DSS-induced Colitis

–Manuscript Draft–

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Abstract:	Inflammatory intestinal disease is characterized by chronic relapsing idiopathic inflammation of the gastro-intestinal tract, persistent inflammation, resulting from poor immunological regulation sustain higher risk for carcinogenesis. Studies focusing on the immune-regulatory function of reactive oxygen species (ROS) and how their deficiency impacts inflammation-mediated tumorigenesis are still largely missing. In this study we developed a new mouse-model of ROS-deficiency leading to colon-adenocarcinoma. Colitis was induced with DSS in wild-type (WT) and Ncf1-mutant (Ncf1*) B10.Q mice using two different protocols, one mimicking recovery after acute colitis (P1) and another simulating a chronic colitis (P2). Disease progression was monitored through evaluation of clinical parameters. For each experimental time-point colon was removed for histopathological analysis. Clinical scores (weight lost, stool consistency, blood in stools, colon length and spleen weight) were significantly worst in Ncf1* than WT-mice. Ncf1* mice with only 7 days exposure to 3% DSS in drinking water followed by 14 days resting period developed colonic high-grade dysplasia when compared with low-grade dysplasia found in the colon of WT mice. Furthermore after the 3 weeks resting period, we observed lower inflammatory reparation in Ncf1* together with high-grade dysplasia and invasive adenocarcinoma, while in WT high-grade dysplasia was prominent without malignant invasion. The results point-out this animal model as a novel tool to study chronic colitis-induced carcinogenesis. ROS-influence in the studied carcinogenesis was underlined by the morphological alterations that were more prominent in Ncf1* mice, whereas the presence of ROS allowed mucosal adaptation in WT mice.
Suggested Reviewers:	

Chapter 7

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7. References

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